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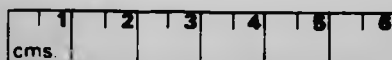
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PLASMID MAINTENANCE IN ESCHERICHIA COLI K-12

A thesis presented by

PAUL DERBYSHIRE, B.Sc.

In part fulfilment of the requirements for
admittance to the Degree of

DOCTOR OF PHILOSOPHY

in the

University of Warwick



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DEDICATION

To my parents Gerald and Irene, my sister Lorraine, and my brother-in-law Eric, in appreciation of their continued support, encouragement and love.

Paul.

ABBREVIATIONS

Ap ^R	resistance to ampicillin.
Ap ^S	sensitivity to ampicillin.
A-T	adenine - thymine.
ATP	adenosine-5'-triphosphate.
ATPase	adenosine triphosphatase.
bp	base pair.
°C	degrees centigrade.
cm	centimetre.
Cm ^R	resistance to chloramphenicol.
C-period	period of chromosome replication during the <u>E.coli</u> cell cycle.
CsCl-EtBr	caesium chloride-ethidium bromide.
c-terminal	carboxy-terminal.
c-terminus	carboxy-terminus.
D	dilution rate.
DNA	deoxyribonucleic acid.
DNase	deoxyribonuclease.
dNMP	deoxynucleotide monophosphate.
EDTA	Ethylenediaminetetraacetic acid disodium salt.
g	gram
G-C	guanine-cytosine.
GDH	glutamate dehydrogenase.
GOGAT	glutamate synthase.
GS	glutamine synthetase.
hr(s)	hour(s)
H-strand	heavy strand (synthesized strand).
HTP	<u>E.coli</u> heat shock regulon.
Iel	immunity to colicin El.
kb	kilobase pair.
kDa	kilodalton.
K _m	Michaelis-Menten constant.
Km ^R	resistance to kanamycin.
l	litre.
L-strand	light-strand (template strand).

abbreviations con't.

m	metre.
M	molar.
MDa	megadalton.
mg	milligram
min(s)	minute(s).
ml	millilitre.
mm	millimetre.
mM	millimolar.
MMS	methyl methanesulphonate.
mRNA	messenger ribonucleic acid.
NAD(P)	Nicotinamide adenine dinucleotide phosphate.
NAD(P)H	Nicotinamide adenine dinucleotide phosphate reduced form.
nm	nanometre.
3'-OH	3'-hydroxyl.
pfu	plaque forming units.
Pi	inorganic phosphate.
Pit	low-affinity inorganic phosphate transport system.
PL	DL-pantoylactone.
PR	partition related segment.
psi	pounds per square inch.
Pst	high-affinity phosphate-specific transport system.
r-determinant	resistance-determinant.
REP	repetitive extragenic palindromic sequence.
RNA	ribonucleic acid.
RNase	ribonuclease.
RNAase H	ribonuclease H
rpm	revolutions per minute.
RTF	resistance transfer factor.
sec(s)	second(s)
Sm ^R	resistance to streptomycin.
SOS	<u>E.coli</u> distress response regulon.
Su ^R	resistance to sulphonamides.
Tc ^R	resistance to tetracycline.
Tc ^S	sensitivity to tetracycline.
tris	tris (hydroxymethyl)-methylamine.
t-RNA	transfer ribonucleic acid

abbreviations con't.

μg	microgram.
μl	microlitre
μM	micromolar.
μm	micron.
μm^3	cubic micrometre.
U.V.	ultra violet.
V	volt
vol(s)	volume(s)
v/v	volume to volume ratio.
w/v	weight to volume ratio.

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PREFACE

As the reader will become aware, the study of plasmid maintenance in E.coli K-12 links two very broad and complicated areas of molecular genetics, namely plasmid and cellular biology. I have attempted to present sufficient coverage of both these areas, to allow adequate interpretation of the results presented, this remains the unavoidable reason for the length of this thesis. I have endeavoured to limit the contents to a minimum, however, you the reader will be the judge of this. Hence, for any seemingly irrelevant or lengthy passages, I apologise.

Paul Derbyshire.

SUMMARY

This study investigated the maintenance of several plasmid derivatives of pBR322 and ColE1 present within Escherichia coli K-12, with a view to establishing a strategy that would ensure the stable maintenance of plasmid pBR322 and the identification of a postulated partitioning function on plasmid ColE1.

Two strategies were adopted. The first of these used selective nitrogen-limited chemostat culture of a glutamate-dependent strain of E.coli, carrying a pBR322 derivative expressing glutamate dehydrogenase. Using this approach, it was found that the plasmid conferred a reproductive advantage and persisted, during nitrogen limitation, for several generations beyond that of pBR322 under glucose- or phosphate-limited conditions.

The second strategy used nonselective chemostat culture of a strain of E.coli carrying derivatives of pBR322 encoding stability functions from either plasmid pSC101, the partitioning region par, or plasmid R1, the cell division/plasmid inheritance coupling region parB. Although these plasmids persisted for many generations beyond that of pBR322 under similar chemostat culture conditions, no conclusions could be made with respect to the ability of these functions to ensure stable plasmid maintenance, since the copy numbers of the respective plasmids were several-fold greater than that of pBR322, a factor that in itself would contribute to the segregational stability of the plasmids. Plasmid-free segregants which did arise were found not to be isogenic with the host strain. These mutants exhibited an increased resistance to U.V.-light irradiation, a mucoid colony phenotype, an altered cell division cycle, giving rise to minicell, filament and Y-shaped cellular morphologies, an enhanced ability to form tandemly repeated plasmid multimers and an altered sensitivity to the DNA gyrase specific antibiotics novobiocin and nalidixic acid. A study of plasmid configuration in lon and lon sul strains of E.coli, which share similar phenotypic characteristics with the above mutants, revealed that strains which carry a sul or azi mutation express an enhanced capacity for plasmid multimerization.

Finally, no conclusive evidence could be obtained to indicate the presence of a partitioning function on plasmid ColE1.

This study concludes by postulating several factors which may affect the maintenance of plasmids in E.coli K-12.

CHAPTER I

REVIEW

1.1 Introduction

All essential genetic information in the bacterial cell normally resides on the chromosome, however, in addition to the chromosome bacteria often contain other covalently closed circular double-stranded DNA molecules, known as plasmids. Plasmids replicate, by using some or all of the chromosomally encoded replication functions, however, plasmid replication is independent of that of the chromosome. Plasmids, therefore, carry information for the control of their own maintenance, these maintenance control functions define the plasmid as a replicon (i.e., an autonomous unit of replication), and together with physiological conditions and factors encoded by the host, these replication control functions determine the number of plasmid replicons per host genome equivalent, termed plasmid copy number.

Plasmids have been found to be widely distributed within prokaryotic species and are capable of conferring new phenotypes on their host cell (Table 1.1). Some of the phenotypic traits exhibited by plasmid-borne genes include antibiotic resistance, antibiotic production, sugar fermentation, heavy metal resistance, bacteriocin production and iron transport. These plasmid-borne traits are normally dispensible except in certain selective environments (i.e., the selection of antibiotic resistance in the presence of antibiotics).

<u>Plasmid</u>	<u>Original host</u>	<u>Molecular weight</u> (MDa)	<u>Copy No.</u>	<u>Conjugative</u>	<u>Characteristics</u>
ColEI-K30	<u>Escherichia coli</u>	4.2	10-15	NO	Colicin EI production.
ClodF13	<u>Enterobacter cloacae</u>	6.0	10	NO	Cloacin DF13 production.
pSC101	<u>Salmonella panama</u>	6.0	5	NO	Tetracycline resistance.
R6K	<u>Proteus rettgeri</u>	25.0	12-40	YES	Ampicillin and Streptomycin resistance.
RP1	<u>Pseudomonas aeruginosa</u>	38.0	1-2	YES	Multiple drug resistance (broad host range plasmid).
R1	<u>Salmonella paratyphi</u>	58.0	1-2	YES	Multiple drug resistance.
RI00	<u>Shigella flexneri</u>	58.0	1-2	YES	Multiple drug resistance.
F	<u>Escherichia coli</u> K-12	63.0	1-2	YES	Integrates to form Hfr strains and can form F' plasmids.
R6	<u>Escherichia coli</u>	66.0	1-2	YES	Multiple drug resistance.
ColV-K30	<u>Escherichia coli</u>	70.0	1-2	YES	Colicin V production and aerobactin-mediated iron transport.
ColV, I-K94	<u>Escherichia coli</u>	85.0	1-2	YES	Colicin V and I production and complement resistance.

Table 1.1

Properties of a range of conjugative and nonconjugative plasmids in bacteria. A double-stranded DNA molecule comprising 1500bp has a molecular weight of about 11MDa, and 1000 bases codes for a polypeptide of about 333 amino acid residues, molecular weight about 30kDa.

Plasmids can vary in size from 1 megadalton (MDa), to greater than 200MDa, and may be conveniently categorized into one of two major groups, conjugative or nonconjugative, depending on whether or not they encode a set of genes designated tra, which permits bacterial conjugation (a process promoted by conjugative plasmids, whereby genetic material is transferred from one bacterium to another via a physical connection). Nonconjugative plasmids cannot promote their own conjugal transfer, however, some nonconjugative plasmids in the presence of an unrelated conjugative plasmid can be mobilized to a recipient cell. In general, conjugative plasmids are large (greater than 40MDa), and exist at 1-2 copies per host genome equivalent, whereas the majority of nonconjugative plasmids tend to be of low molecular weight and may be present at multiple copies (Table 1.1). There are, however, exceptions to the above generalization, for example, the conjugative plasmid R6K has a molecular weight of 25MDa but is maintained at a relatively high copy number, while the 6MDa nonconjugative plasmid pSC101 is maintained at only a few copies.

The study of plasmid biology has in recent years resulted in a number of significant developments that have contributed to the discipline of molecular biology as a whole. For instance, the exploitation of plasmids as cloning vehicles has enabled the convenient study and commercialization of cloned genes and their products. Furthermore, the close association of plasmids with bacterial pathogenicity, has widened our understanding

as to the importance of extrachromosomal elements in both the treatment and underlying causes of bacterial disease. Not surprisingly, such insights into plasmid biology has posed questions concerning the nature of both plasmid propagation and inheritance within bacterial populations. For the time being it will be useful to briefly overview some fundamental aspects relating to plasmid biology, this will then be followed by a section which attempts to outline some of the reasoning behind the subject areas which have been chosen for a more detailed review, together with their relevance to the study of plasmid maintenance.

1.1.1 Plasmid incompatibility

Plasmid incompatibility defines the inability of two closely related plasmids, in the absence of continued selective pressure, to co-exist in the same cell. Incompatibility is a function of the control of plasmid maintenance and can be a measure of the relatedness between the same maintenance functions of different plasmids (Novick & Hoppensteadt, 1978). Analysis of plasmid incompatibility has indicated that it primarily derives from two functions, a) control of plasmid replication, and b) partitioning (partitioning refers to the physical distribution of plasmid molecules between daughter cells during cell division), (Ishii et al., 1978; Novick & Hoppensteadt, 1978). Plasmid functions associated with the control of plasmid replication appear to exert the strongest incompatibility effects, and two models have been proposed to describe incompati-

bility occurring as a consequence of replication control. The first model interprets incompatibility as being the consequence of competition for a common membrane maintenance site (positive control model of Jacob et al., 1963). Whilst the second model proposes that a repressor encoded by one plasmid interferes with the replication of another plasmid within the same cell (negative control model of Pritchard et al., 1969). All plasmids studied so far appear to negatively control their own replication. Indeed, variations in the copy number of incompatible plasmids has been shown to lead to a reciprocal variation in the copy number of incompatible co-resident plasmids (Projan & Novick, 1984). Furthermore, the constant frequency at which monoplasmid containing segregants appear during growth and division of a polyplasmid containing population of cells, has been demonstrated to be inversely proportional to the initial copy number of the displaced plasmid (Novick & Hoppensteadt, 1978). This form of incompatibility has been suggested as being the result of two plasmids occupying a common copy number pool, that is, these plasmids have a common negatively controlled copy number mechanism which determines the total plasmid copy number within a cell.

Such observations regarding the phenomenon of incompatibility have suggested the occurrence of two random independent processes, one in the choice of replicon for plasmid replication and the other in the selection of individual molecules for partitioning (Ishii et al., 1978). Plasmid partition functions,

however, do not always exhibit incompatibility; as is the case with plasmid pSC101 (Nordstrom et al., 1980b). When incompatibility is observed between partition functions, as shown by plasmid R1 (Nordstrom et al., 1980b), then there is only a weak effect, which suggests that plasmid incompatibility is a phenomenon mainly associated with plasmid replication control functions. However, plasmid incompatibility is a complex phenomenon and may involve several different plasmid-encoded systems.

1.1.2 Plasmid replication

Two mechanisms have been proposed to account for the replication of plasmid DNA. The first of these models relates to vegetative replication, that is the normal synthesis of plasmid DNA, and is based on the observations of Cairns who used gentle lysis techniques to obtain intact DNA molecules for autoradiographic analysis (Cairns, 1963). Cairns experimental approach has suggested that DNA replication is initiated at a fixed point, termed oriV, and proceeds either bidirectionally, as is the case for the E.coli genome and some conjugative plasmids such as F, or as for most plasmids unidirectionally to a defined terminus (Fig.1.1A). Hence, both strands of a circular DNA duplex are replicated without initial strand breakage, strands are separated forming a theta structure as replication proceeds. The second mode of plasmid replication may describe events which occur during plasmid transfer in conjugation or mobilization, that is

Figure 1.1

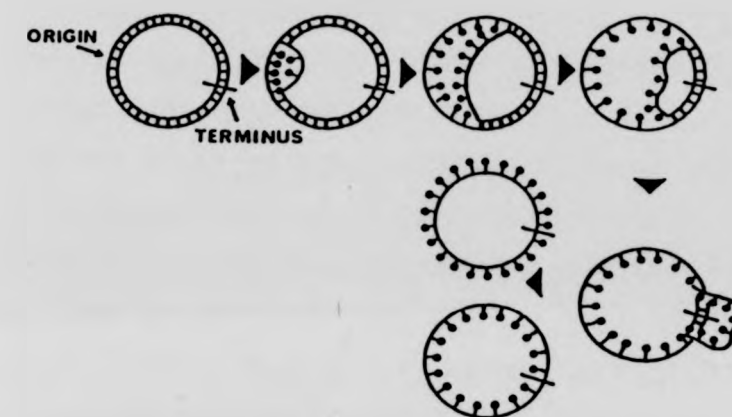
Schematic representation of mechanisms for the replication of covalently closed circular DNA molecules.

A) Cairns-type replication, represented as bidirectional replication initiating at the origin, the respective replication forks proceed to a terminating point diametrically opposite the origin.

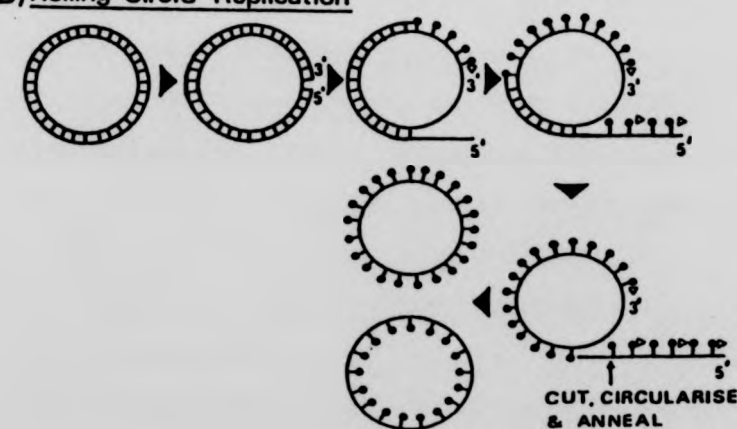
B) Rolling circle replication, this is initiated with cleavage of one strand of the DNA duplex, the resultant 3'-OH end functions as a primer for continuous DNA synthesis, while replication on the displaced strand is discontinuous (as represented by open arrowheads). Replication on the displaced strand results in concatemeric lengths of DNA which must be cleaved and circularized to form duplicated unit circular molecules.

(Reproduced from Gene function, E.coli and its inheritable elements, by R.E. Glass, Croom Helm, London, 1982).

A) Cairns-type Replication



B) Rolling Circle Replication



transfer replication. Termed the 'rolling-circle' model (Fig.1.1B), this mode of replication is initiated when one strand of the DNA duplex is cleaved or 'nicked' at the transfer origin, oriT (which is distinct from the vegetative site oriV), and is displaced from the intact strand during discontinuous replication, the intact strand serves as a template for the continuous synthesis of the complementary displaced strand.

Since the study of DNA synthesis in large replicons such as the E.coli genome is difficult, replication mechanisms of extrachromosomal genetic elements have been utilized as model systems for the study of DNA synthesis. Indeed, there is an overall similarity between the replication of chromosomal and extra-chromosomal genetic elements, indicating that at least the mechanisms by which discontinuous and possibly continuous DNA synthesis occurs may be very similar (Wickner, 1978; Kornberg, 1980). However, dependence on the E.coli host-encoded initiation factor DnaA, with the exception of pSC101, appears to be unique for replication initiated at the E.coli origin, oriC (Hasunuma & Sekiguchi, 1977).

All plasmids studied to date negatively control their own DNA replication and this may be accomplished by one of three different mechanisms in which the main inhibitor of plasmid replication can either be a protein, as is the case in lambda dv (Matsubara, 1981), a small RNA, as is the case for plasmids ColE1 (Tomizawa et al., 1981), and CloDF13 (Stuitje et al., 1981), or a series of direct DNA repeats, as may be the case for

plasmid pSC101 (Linder et al., 1985), the F plasmid and prophage P1 (Chattoraj et al., 1984 & 1985). It appears, therefore, that the initiation of DNA synthesis rather than chain elongation is the stage at which these elements primarily differ. Indeed, control of the frequency of plasmid replication events may be expected to be exerted at the initiation stage of each new round of DNA synthesis, which would suggest that regulation of initiation of plasmid DNA synthesis is synonymous with the control of plasmid copy number (Scott, 1984).

Finally, certain plasmids in the presence of a translational inhibitor, such as chloramphenicol, are able to continue plasmid replication resulting in a several-fold amplification of plasmid copy number, a phenomenon that has been termed 'relaxed-replication'. This is in contrast to other plasmids whose replication under similar conditions is inhibited; their replication has been termed 'stringent'.

1.1.3 Plasmid partitioning

Extrachromosomal replicons may be distributed to progeny cells either via an active or passive segregation process. In the case of passive segregation plasmid inheritance relies on the random distribution of plasmid molecules within the mother cell. The probability $P(o)$ of either daughter cell failing to inherit a plasmid is given by the binominal distribution $P(o) = 2(\frac{1}{2})^c$, where c is the number of plasmid molecules prior to division (Meacock & Cohen, 1980). For plasmid molecules normally present at a high-copy number, a low

frequency of plasmid-free cells would be expected, while for plasmids present at a low-copy number, the frequency of plasmid free cells would be high (Table 1.2). This random process of segregation, however, is in addition dependent on functions that control the frequency of initiation of plasmid replication. Any reduction of plasmid-copy number in progeny cells, produced at division by the stochastic process, must be corrected prior to the production of the next generation of daughter cells. Plasmids, therefore, that exist at a sufficiently low-copy number will have a high probability of producing plasmid-free cells, unless there is specified a system(s), either plasmid or chromosomally encoded, enabling active partitioning.

Plasmid-encoded partitioning systems, normally designated par, have been described for several low-copy number plasmids such as F (Ogura & Hiraga, 1983a), pSC101 (Meacock & Cohen, 1980), and the bacteriophage P1, which exists as a unit-copy plasmid in the prophage state (Austin & Abeles, 1983b). In addition, deletion derivatives of plasmids R1 (Nordstrom *et al.*, 1980a), NR1 (Miki *et al.*, 1980), and Rms201 (Ike *et al.*, 1981a), which are defective in segregation, have been isolated and may be deficient in plasmid-encoded par functions. However, the above evidence does not exclude the possibility that host specified systems may also operate.

One of the best characterized and most simple of these par systems appears to be that encoded by plasmid pSC101, which consists of a cis-acting locus adjacent

<u>Plasmid copy number (c)</u>	<u>Probability (P(0))</u>
1	1
2	5.00×10^{-1}
3	2.50×10^{-1}
4	1.25×10^{-1}
5	6.20×10^{-2}
10	1.95×10^{-3}
20	1.91×10^{-6}
30	1.86×10^{-9}
40	1.82×10^{-12}

Table 1.2

Probabilities of segregating plasmid-free progeny in relation to plasmid copy number, if no active plasmid partitioning system exists.

to, but independent of the origin of replication of the plasmid (Meacock & Cohen, 1980). The most elaborate par mechanism described is that for the F plasmid, located within a 3 kilobase pair (kb), region adjacent to, but likewise independent of the region essential for F replication. In the case of the F plasmid both trans- and cis-acting factors are involved (Ogura & Hiraga, 1983a), and a similar system has also been described for the par determinant of bacteriophage P1 (Austin & Abeles, 1983b).

Models which have been put forward to describe plasmid partitioning events basically differ with respect to whether plasmids are equipartitioned, that is, each daughter cell receives half the number of plasmid copies present at cell division, or whether one plasmid copy is distributed to each daughter cell the remaining plasmid copies being randomly distributed. One way of testing these models has been to use replication temperature-sensitive plasmids. The 'equipartition' model as described by Novick et al. (1975), was derived from conclusions based on temperature shift experiments with a population of cells harbouring a temperature-sensitive, repts, Staphylococcus aureus plasmid. Results suggested that plasmid-free cells did not appear until pre-existing copies of the nonreplicating plasmid were reduced to one copy per cell by an equipartition mechanism. Similar results have been reported for plasmids pSC101 (Hashimotoh-Gotoh & Sekiguchi, 1977), and Rms201 (Ike et al., 1981b). However, the frequency

of segregation of plasmid-free cells in the above experiments were estimated from the difference between viable counts on antibiotic-free and antibiotic-containing medium.

Recent studies of plasmid segregation, involving a more accurate measurement of plasmid-free cells, and the use of a temperature-sensitive replication mutant of pSC101, has lead to the development of a model for replicon duplication and partitioning (Hashimoto-Gotoh & Ishii, 1982). This model, termed the 'single-site inheritance' model, postulates the occurrence of both random and active partitioning and assumes that for each plasmid replication event, a single plasmid molecule is employed as a template at a replicon-specific membrane site. At cell division it is the two nascent plasmid copies present at this single site which are actively partitioned to the daughter cells, whilst plasmid molecules residing in the cytoplasm are randomly distributed. This particular model implies that there is a coupling between plasmid replication and partitioning. However, an alternative random partition model, based on calculations and experiments with plasmid R1, has been proposed. This model is similar to the 'single-site inheritance' model but does not assume a link between plasmid replication and partitioning (Nordstrom & Aagaard-Hansen, 1984). This alternative mechanism of partitioning, termed the 'pair site' model, assumes that a plasmid pair are chosen for partitioning at the 'pair site', possibly located within the cell membrane, whilst plasmid copies present within the

cytoplasm are randomly distributed. The 'pair site' and 'single-site inheritance' models may therefore be expected to differ with respect to their incompatibility properties. It is interesting to note that the par loci of both plasmids pSC101 and R1 have been shown to specifically associate with membrane fractions, having the same density as the outer membrane of E.coli K-12, when separated and analysed in sucrose gradients (Gustafsson et al., 1983).

Unfortunately, as expanded on recently (Nordstrom, 1984), these models do not take into account variations in plasmid copy number distribution within a bacterial population, or cell size distribution at cell division. Therefore, kinetics of plasmid segregation cannot readily distinguish between either equipartition, or more random modes of plasmid partition.

1.1.4 Cell division coupling to plasmid replication

Studies involving plasmid R1 have indicated that within a steady-state culture the presence of R1 affects the pattern of cell division in a proportion of the population, leading to filament formation (Engberg et al., 1975). Recently, a system that inhibits cell division in cells carrying only one plasmid copy has been described for the F plasmid (Ogura & Hiraga, 1983b). The ccd system (control of cell division), of the F plasmid, may operate by inducing some component of the cells SOS response, one function of which is to inhibit the cell division process.

1.1.5 Plasmid recombination

Plasmid multimerization has been postulated as a factor that reduces, or interferes with the efficiency of plasmid partitioning (Fig.1.2), (Austin et al., 1981). Mechanisms of plasmid replication control suggest that it is the number of vegetative replication origins per host cell which is fixed. Indeed, it has been shown that for ColE1-related plasmids, copy number control is determined by a process of 'origin counting' (Summers & Sherratt, 1984). The formation of plasmid multimers would be expected to result in the same number of replication origins, but fewer plasmid molecules present per cell, leading to a reduction in the number of plasmid molecules available for distribution at cell division.

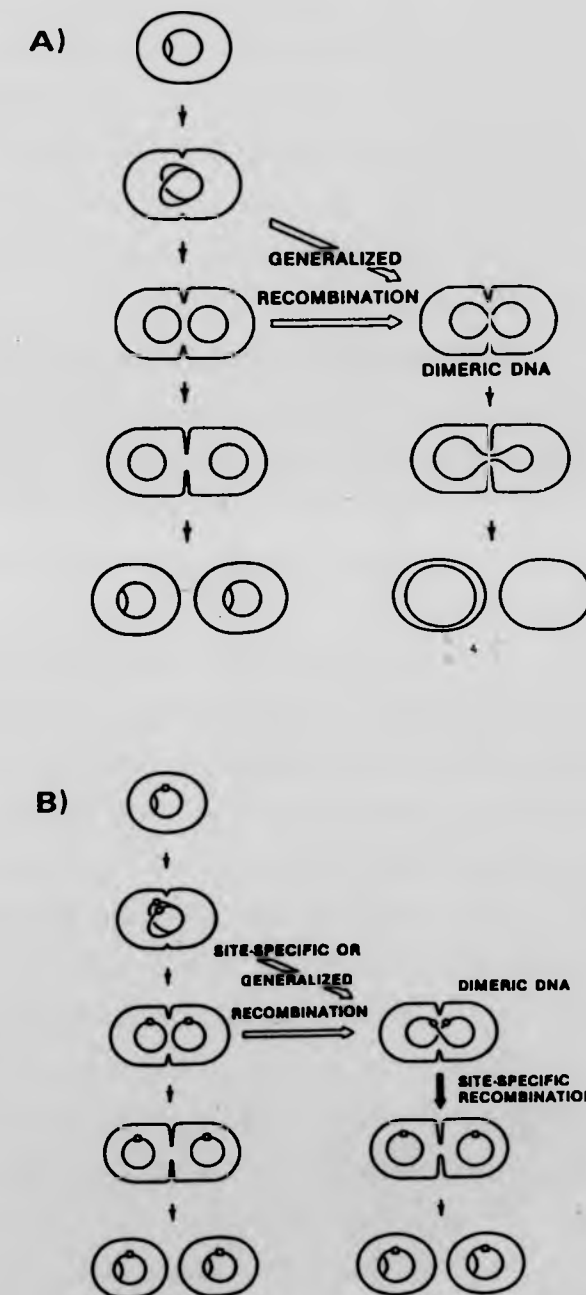
Plasmid-encoded site-specific recombination systems have been described which appear to limit the extent of multimer formation. The unit-copy plasmid P1 encodes both a cis-acting locus and a trans-acting recombinase, which mediate co-integrate resolution of P1 multimers (Austin et al., 1981). However, bacteriophage P1 also specifies an inversion sequence which governs the host range of the phage. This inversion system is also capable of generating plasmid P1 multimers in the absence of the site-specific recombination system (Kennedy et al., 1983). Plasmids ColE1 and CloDF13 have also been shown to encode cis-acting loci necessary for co-integrate resolution. However, they are presumed to rely on a specific host-encoded protein(s), to catalyse intraplasmidic recombination (Summers &

Figure 1.2

Diagrammatic representation of the postulated effects of plasmid multimer formation and resolution on plasmid partitioning.

- A) Generalized recombination leading to dimer formation of a unit-copy replicon. Plasmid-free segregants arise as a consequence of dimer formation (right pathway), blocking the normal pathway for plasmid partitioning (left pathway).
- B) The extent of dimer formation is restricted by the presence of a site-specific recombination system (right pathway), resolving dimers to monomers and providing unit-copy replicon substrates for plasmid partitioning.

(Reproduced from Austin *et al.*, 1981).



Sherratt, 1984; Hakkaart et al., 1984).

Finally, it must be noted that in contrast to reducing the efficiency with which a randomly partitioned plasmid is inherited, recombination events may lead to the formation of co-integrates with either the host chromosome or a plasmid containing a par locus, thereby ensuring plasmid inheritance.

1.1.6 Summary and rationale for review

To summarize, it would appear that the stable maintenance of bacterial plasmids is affected by at least four different maintenance functions,

a) replication and copy number control,

b) partitioning,

c) control of host cell division, and

d) site-specific recombination. Plasmids that are maintained at a low-copy number would appear to require at least three of the above functions, so as to be stably maintained. Furthermore, these systems appear to function independently of one another and various combinations may be utilized by any particular plasmid.

By implication from this brief overview of plasmid maintenance, the subject area of this study covers a number of diverse disciplines relating to the molecular biology of the E.coli cell. Primarily, plasmid maintenance requires an understanding of the different modes of both plasmid replication control and partitioning. For this purpose, I will categorize plasmids as being either high-, intermediate-, or of unit-copy number, since as will become apparent, the

complexity of plasmid-encoded maintenance functions appears to correlate directly with copy number maintenance. For this reason I have chosen to review well studied plasmids representative of each copy number category. Since experimentation carried out in this study involved the use of ColE1-type replicons, plasmids ColE1, pBR322 and CloDF13 have been chosen to represent high-copy number plasmids. Similarly, since a part of this study involved the experimental use of the par region, derived from plasmid pSC101, this plasmid has been chosen to represent intermediate-copy number plasmids. For unit-copy number plasmids, both F and P1 are described, as is plasmid R1, since a part of this study involved the experimental use of the parB region of R1.

The segregation of genetic material between daughter cells at cell division is central to the cell division process. Within an expanding bacterial population, derived from a clonal cell, genetic information must be faithfully duplicated and at least one copy of the information, including that which is plasmid-encoded, be transferred to each new-born cell. For this reason it has been necessary to review aspects of the control of cell division in E.coli K-12, and in particular, responses to perturbations in DNA synthesis. Pathways of recombination, particularly those affecting plasmid recombination are also described.

Finally, since this study makes use of the techniques of continuous culture, as a principle method of investigating and demonstrating the sometimes

difficult phenotype of plasmid stability, a section of this review is devoted to a current understanding of the use of continuous culture in the study of plasmid stability.

1.2 Plasmid replication and partitioning

1.2.1 Plasmid ColE1

a) Introduction

ColE1 is a naturally occurring plasmid, existing under non-limiting nutrient conditions at a copy number of about 15-20. It replicates independently from the host chromosome, can be mobilized in the presence of some conjugative plasmids, and codes for the colicinogenic protein colicin E1, as well as a protein conferring host immunity to the colicin (Fig.1.3), (Veltkamp and Stuitje, 1981).

b) Plasmid copy number control

For ColE1-type replicons, initiation of plasmid replication is negatively regulated by two plasmid encoded gene products, a small RNA transcript of about 108-110 nucleotides in length designated RNAI (Tomizawa et al., 1981), and Rop a 63 amino acid polypeptide (Cesareni et al., 1982). A second RNA transcript initiating at 555 base pair (bp), upstream from the origin of replication, when processed by Rnase H, serves as a primer for DNA polymerase I initiated plasmid replication (Fig.1.4), (Itoh & Tomizawa, 1980; Masukata & Tomizawa, 1984). The formation of a stable hybrid between ColE1 pre-primer RNA

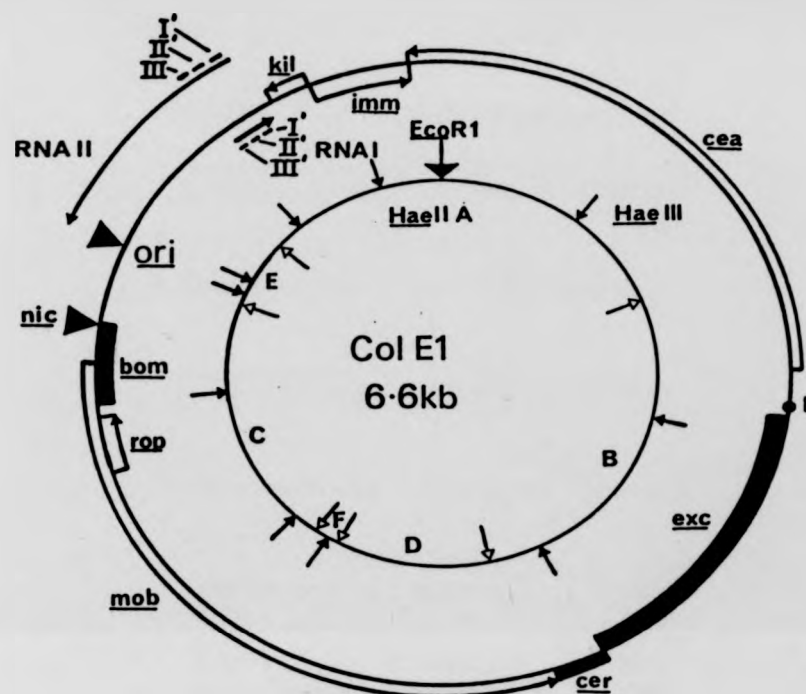


Figure 1.3

Genetic and functional map of plasmid ColE1. The inner circle represents sites at which the restriction enzymes HaeII; ↓, and HaeIII; ↓, cleave, together with the single EcoRI site which provides a reference point on the ColE1 map. The outer circle defines the transcriptional orientation of regions encoding ColE1 functions, for colicin E1 (cea), immunity to colicin E1 (imm), mitomycin C-induced lethality (kil), incompatibility and copy number control (RNAI), primer RNA (RNAII), vegetative origin of replication (ori), transfer origin (nic), basis of mobility (bom), repressor of primer formation (rop), functions necessary for mobilization (mob), locus for colicin E1 site-specific resolution (cer), and functions necessary for plasmid exclusion (exc). (Compiled from Oka & Takanami, 1976; Tomizawa et al., 1977; Dougan et al., 1978; Veltkamp & Stuitje, 1981; Naumora et al., 1981).

PROCESS OF PRIMER FORMATION

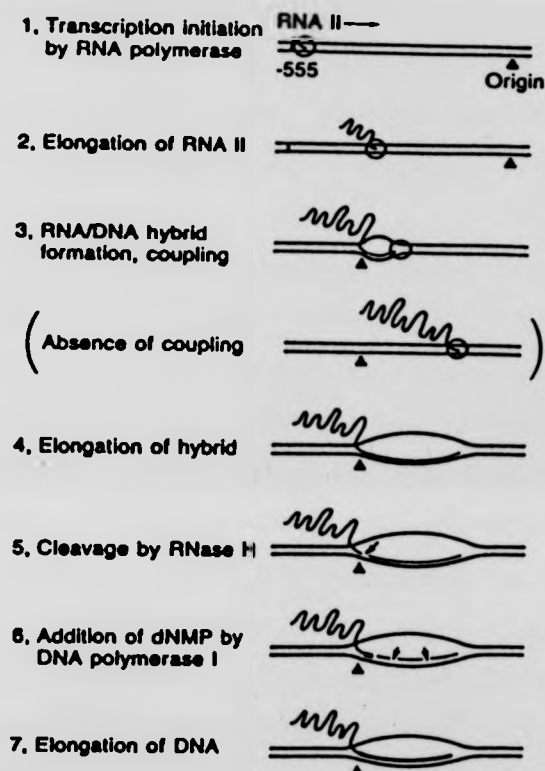


Figure 1.4

Diagrammatic representation of the process of primer (RNAII) formation. Each stage is annotated by a brief description, for stage 3 there are two alternatives, one of which prevents primer formation and is surrounded by brackets. Straight lines, indicate double-stranded DNA, wavy lines, transcript of RNAII, small circles, RNA polymerase, arrows in the eye structures of stages 5 and 6, cleavage of hybridized RNA by RNAase H, and the thick line in stages 6 and 7, represents newly synthesized DNA. (Reproduce from Masukata & Tomizawa, 1984).

and DNA, is essential for the initiation of DNA replication, presumably by allowing RNase H, an enzyme whose activity is specific for RNA:DNA hybrids, to cleave the RNA pre-primer at the origin yielding an RNA primer possessing a 3'-OH end (Fig.1.4), (Masukata & Tomizawa, 1984).

In vitro studies carried out to reconstitute ColEl-type replication, suggest that RNase H may also act as a discriminatory factor inhibiting initiation events occurring at sites other than the origin (Hillenbrand & Standenbauer, 1982). Indeed, a ColEl mutation having a single base pair alteration at 160 nucleotides upstream from the origin, cannot replicate in wild-type cells, but is able to replicate in RNase H deficient cells, suggesting that the secondary structure of the pre-primer RNA:DNA hybrid may be important in protection from and processing by RNase H (Ogawa & Okazaki, 1984). Evidence has also been presented for the involvement of the B subunit of DNA gyrase in ColEl replication, since initiation is sensitive to the degree of superhelicity of the DNA template (Orr & Staudenbauer, 1981).

Transcription of RNAI initiates some 110bp downstream from the promoter for RNAII, and is complementary to the initial 100 or so nucleotides of RNAII. Therefore, any mutational change in the sequence coding for RNAI would result in a complementary change in RNAII. The RNAI molecule can be folded into a structure similar to that of t-RNA, consisting of three stem and loop structures designated I', II' and III', to distinguish them from the complementary hairpin loops I, II and III of RNAII (Fig.

1.5), (Davison, 1984). Pre-primer RNA, is able in vitro, to form a double strand RNA hybrid with RNAI, as demonstrated by the double stranded RNA hybrids sensitivity to RNase III (Scott, 1984). Hybrid interaction between RNAI and RNAII suggests that control of DNA synthesis and incompatibility may occur by the prevention of primer formation (Tomizawa & Itoh, 1981).

Absence of the Rop protein results in a 2- to 5-fold increase in the copy number of ColE1. This polypeptide has been shown to be perfectly conserved between ColE1-related replicons despite base pair substitutions, all of which occur in the third position of the amino acid codon, leading to a neutral mutation (Davison, 1984). The Rop protein does not appear to affect the transcription of RNAI, but does affect transcription of RNAII. This Rop-mediated inhibitory activity on pre-primer transcription, does however, require the presence of RNAI. The site of action of Rop is located between 50-135bp downstream of the RNAII promoter, and it has been suggested that Rop may enhance hybrid formation between RNAI and RNAII (Cesareni et al., 1984). A regulatory model, analogous to that suggested for transcriptional attenuation, has been proposed to account for the effects of the Rop protein on transcription of RNAII. RNAI itself does not inhibit transcription of RNAII. An alternative may be that interaction of Rop and RNAI with RNAII, affects the stability of the pre-primer with respect to ribonuclease degradation (Lacatena et al., 1984).

Analysis of ColE1 point mutations causing an increased copy number or altered incompatibility, have in general,



Figure 1.5

Postulated secondary structure of Colel-RNAI. Indicating loops I', II' and III', together with stem base pairing as represented by dots. The arrows indicate base changes due to known point mutations in RNAI, the numbers represent the specific mutations which are designated as; 1, svir111; 2, inc3; 3, svir19; 4, cop53; 5, svir12; 6, inc2, svir2, svir7, upcl; 7, svir11; 8, svir020; 9, svir3, cop59; 10, inc9 (this refers to the unique nucleotide difference occurring between the RNAI of Colel and PMB1); 11, incl; 12, inc4; 13, cop93; and 14, cop98.

(Reproduced from Davison, 1984).

been found to be located in the sequences encoding the ends of hairpin loops I' and II' of RNAI (Fig.1.5), (Davison, 1984). From analysis of such mutations two models have been put forward to account for ColE1 incompatibility and replication control. The first of these proposes that the loop II' region of RNAI specifies a negative regulator which interacts with sites in both the loop I and II regions of RNAII (Tomizawa & Itoh, 1981). Loops I and II of RNAII are identical in plasmids ColE1, RSF1030 and CloDF13, but in plasmid pMB1 loop I differs by a single base pair (Fig.1.5), (Davison, 1984). Therefore, an interaction is possible between loop II' of RNAI, and either loop I or II of RNAII. Furthermore, loop I-loop II double mutants have an increased plasmid copy number in comparison to single loop mutations, suggesting that an interaction of the negative regulator with both loop I and II is necessary for normal replication control (Tomizawa & Itoh, 1981). Indeed, replication control of pMB1, as judged by a copy number slightly greater than that of ColE1, is presumed to be simpler since the base pair change in loop I presumably reduces the interaction between loop II' and loop I resulting in only partial regulation (Davison, 1984).

The second model extends the first by proposing a more sophisticated secondary structure for RNAI, comprising of an RNAI dimer construction in which the stems of the hairpin structures are hybridized in a complementary antiparallel manner (Fig.1.6), (Davison, 1984). This results in a double stranded clover leaf arrangement in which the single strand loops I', II' and III' all remain

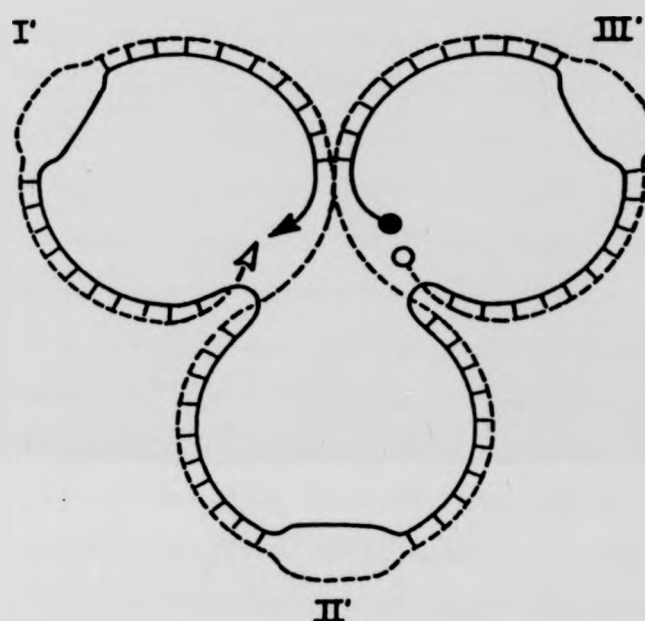


Figure 1.6

Hypothetical secondary structure of a Cole1-RNAI dimer construct. The arrowheads represent the 3'-ends of each molecule, and the lines between the strands represent hydrogen bonding.

(Reproduced from Davison, 1984).

unpaired and available for interaction with RNAII. Indeed, the RNAI dimer structure presents two loop II' negative regulator regions for possible simultaneous interaction with loops I and II of RNAII (Davison, 1984).

The ratio of RNAI to RNAII concentration is also likely to be an important factor affecting ColE1 copy number control. Recent evidence, utilizing RNAI and RNAII promoter fusions to galK (Polaczek & Ciesla, 1984), indicates that the promoter for RNAI at both 30°C and 42°C is some 5-fold more efficient than that of RNAII. In addition, both promoters exhibit a 2-fold increase in activity when shifted from 30°C to 42°C, suggesting that an increase in temperature from 30°C, would be expected to result in a concomitant decrease in plasmid copy number. Furthermore, it was concluded that the E.coli dnaA gene product may act as a repressor of the synthesis of RNAI, since thermal inactivation of DnaA protein resulted in a considerable increase in the activity of the RNAI promoter.

Finally, ColE1 has been shown to associate with the cytoplasmic membrane during chloramphenicol amplification. The ColE1 DNA-membrane complex did not dissociate in the presence of RNase. Polyacrylamide gel electrophoresis indicated that a protein of about 22 kilodalton (kDa), may be involved in complexing ColE1 DNA to the membrane (Clark & Gosier, 1983). Whether this association of ColE1 DNA to form a membrane complex is related to either plasmid replication or partitioning is unknown. However, a DNA-synthesizing complex of about 390kDa, containing DNA polymerase I and exonuclease V, has been isolated. This

complex appears to be stabilized by a component termed factor E, and is associated with membrane fractions via a DNase-sensitive link (Scharff *et al.*, 1983). The presence of exonuclease V within the DNA-synthesizing complex may explain observations concerning exonucleases I, II and V, which appear to be required for the stable maintenance of ColEl-type plasmids. However, this plasmid instability is suppressible by inactivation of the RecF pathway (Inselburg, 1978; Bassett & Kushner, 1984). It has been suggested that both plasmid replication and partitioning could be abnormal in exonuclease I and V deficient cells (Ream *et al.*, 1978).

c) Plasmid stability

Experiments carried out to investigate the stable maintenance of ColEl suggests that only the HaeIIE, and the left half of the HaeIIA fragment are essential (Fig.1.3), (Ohmori & Tomizawa, 1979), and that the presence of the ColEl HaeIIA fragment is necessary for stable plasmid segregation (Inselburg, 1981). It has therefore been concluded that ColEl is stably maintained, since plasmid-free segregants are generally not observed (ColEl has a segregation rate less than 10^{-5} per cell per generation), (Summers & Sherratt, 1984).

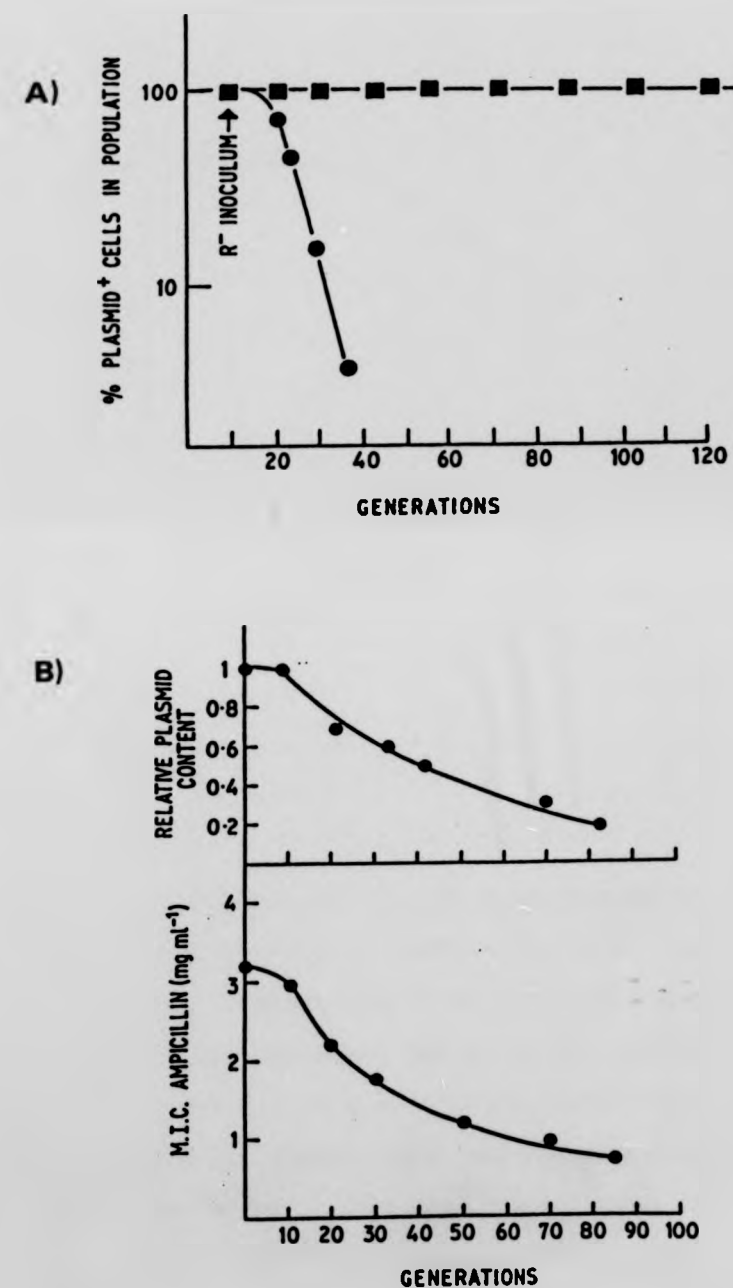
Plasmid RSF2124 is a ColEl::Tn1 derivative possessing a single EcoRI restriction site (Fig.3.14). Recombinant plasmids carrying DNA inserts at this site appear not to produce colicin E1 (So *et al.*, 1975). In order to validate previous observations (Adams *et al.*, 1979), in which colicin E1 production during chemostat culture may

have influenced the outcome of competition experiments between RSF2124-bearing and isogenic plasmid-free E.coli strains, Helling et al. (1981), constructed strains of E.coli harbouring EcoRI generated RSF2124 recombinant derivatives not producing colicin E1. These workers were able to show, using chromosomal mutations that did not affect cellular growth-rate, that a reduction in the proportion of RSF2124-bearing cells during chemostat culture was the consequence of two factors, a) a reduction in the growth-rate of plasmid-bearing cells during nutrient-limited culture, and b) segregational plasmid loss. In a separate study by Jones et al. (1980a), plasmid pUB1246, an RSF2124 derivative containing an EcoRI fragment from plasmid RPl, was likewise shown to be segregationally unstable during chemostat culture. Therefore, observations made using the ColE1 derivative RSF2124, suggest that insertion of EcoRI fragments into the unique EcoRI site of ColE1, leads to the segregational instability of the recombinant plasmid during chemostat culture, and that colicin E1 production may account for the observed stable maintenance of plasmid ColE1.

However, the ColE1 derivative pDS1109 (Fig.3.14), which possesses a Tn1 insertion within the colicin E1 coding region, and therefore fails to produce colicin E1 whilst providing a suitable selectable marker for chemostat studies (beta-lactamase production), (Dougan & Sherratt, 1977; Shafferman et al., 1979), is stably maintained during non-selective phosphate-limited chemostat culture, despite an observed 5-fold reduction in plasmid DNA content (Fig.1.7), (Jones et al., 1980b).

Figure 1.7

- A) Persistence of plasmid pDS1109 in *E.coli* K-12 strain W5445 during phosphate-limited chemostat culture. Other culture parameters were temperature 37°C and a dilution rate of 0.2hr⁻¹ (a mean generation time of about 3.47hrs), the results are presented as a semi-logarithmic plot of percentage ampicillin-resistant population (% Plasmid⁺ cells), with number of generations; ■—■, maintenance of pDS1109 in the absence of a plasmid-free host cell inoculum; ●—●, loss of pDS1109-containing cells on addition of a 0.1% inoculum of isogenic plasmid-free cells. (Reproduced from Primrose *et al.*, 1984).
- B) Changes in the copy number of pDS1109 during glucose-limited chemostat culture of pDS1109-containing cells of *E.coli* K-12 strain W5445. The relative copy number was estimated by measuring the intensity of plasmid DNA following agarose gel electrophoresis of whole cell lysates (upper figure), and the minimum inhibitory concentration for ampicillin (lower figure). Data is reproduced from Jones *et al.* (1980b).



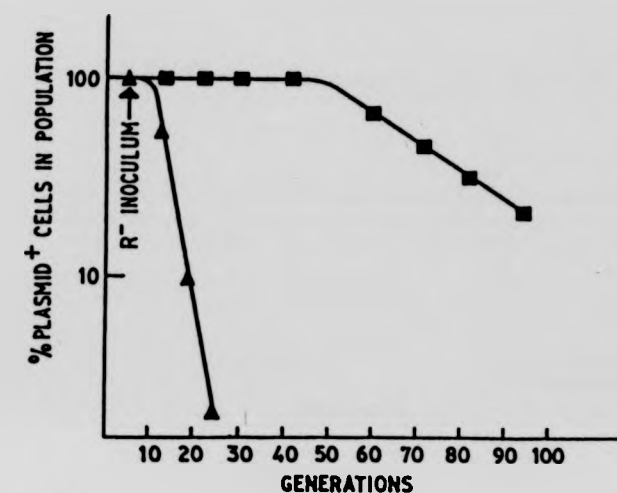


Figure 1.8

Persistence of plasmid pBR322 in *E. coli* K-12 strain W5445 during phosphate-limited chemostat culture. Other culture parameters were, temperature 37°C and a dilution rate of 0.2hr^{-1} (a mean generation time of about 3.47hrs), the results are presented as a semi-logarithmic plot of percentage ampicillin-resistant population with number of generations; ■—■, segregation of pBR322-free cells; ▲—▲, accumulation of plasmid-free cells on addition of a 0.1% inoculum of isogenic plasmid-free cells. (Reproduced from Primrose *et al.*, 1984).

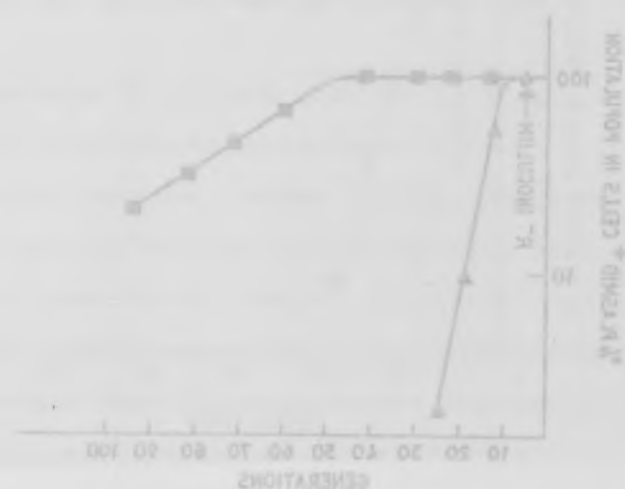


Figure 1.8

Persistence of plasmid pBR322 in *E. coli* A-11 strains W3445 during phosphate-limited chemostat culture. Other culture parameters were: temperature 37°C and a dilution rate of 0.2hr⁻¹ (a mean generation time of about 3.4hrs). The results are presented as a semi-logarithmic plot of percentage ampicillin-resistant population with number of generations; —●—, segregation of pBR322-free cells; —▲—, accumulation of plasmid-free cells on addition of a 0.1% inoculum of isogenic plasmid-free cells. (Reproduced from Primrose *et al.*, 1984).

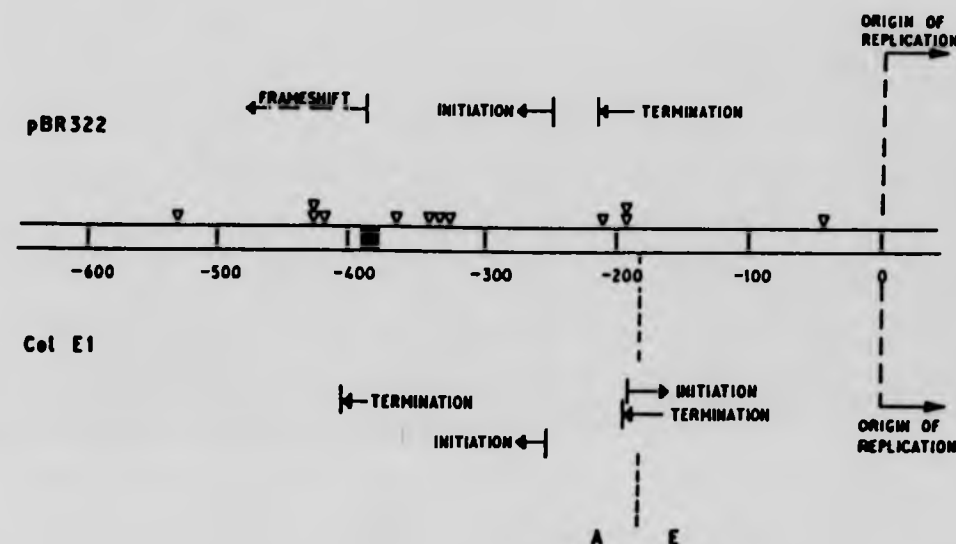


Figure 1.9

Comparison of the DNA sequences of plasmids ColE1 and pBR322, upstream from the origins of their replication. The numbers represent the distance in nucleotide pairs from the origins of replication. The arrowheads indicate the positions of single base pair changes. The closed box, represents a region in which a base pair sequence in ColE1, is replaced by an unrelated 10 base pair sequence in pBR322. The potential sites of initiation, or termination of translation (protein synthesis), are indicated, as is the junction of the *Hae*IIA and E fragments of ColE1. (Reproduced from Primrose *et al.*, 1984).

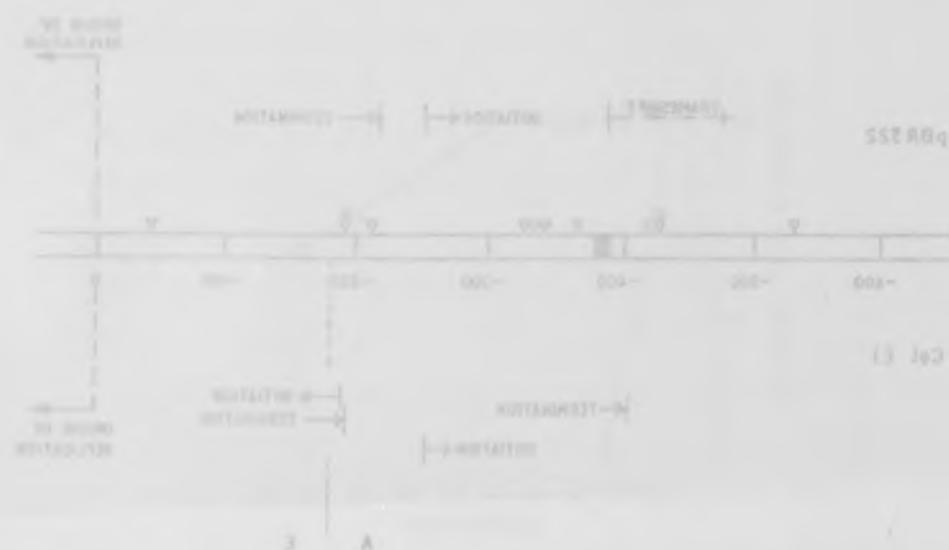


Figure 1.3

Comparison of the DNA sequences of plasmids ColE1 and pBR322, upstream from the origins of their replication. The numbers represent the distance in nucleotide pairs from the origins of replication. The arrows indicate the positions of single base pair changes. The closed box represents a region in which a base pair sequence in ColE1 is replaced by an unrelated 18 base pair sequence in pBR322. The potential sites of initiation, or termination of translation (protein synthesis), are indicated, as is the junction of the *Hae*III and *E* fragments of ColE1. (Reproduced from *Primrose et al.*, 1984).

Furthermore, pDS1109 did not confer a selective advantage on the host strain, since the introduction of a 0.1% inoculum of isogenic plasmid-free cells rapidly predominated over the pDS1109-bearing cells (Fig.1.7A). In contrast, the related plasmid pBR322, which contains a ColE1-type replication region, was not stably maintained under similar conditions of chemostat culture (Fig.1.8), (Jones *et al.*, 1980b). It was therefore concluded that pDS1109 encodes a function necessary for the fidelity of ColE1 segregation during cell division that is absent in pBR322 and certain derivatives of RSF2124.

As mentioned above, the replication region of pBR322 is related to that of ColE1, a comparison of their DNA sequences may highlight regions with sufficient differences to account for the instability of pBR322 (Fig.1.9), (Oka *et al.*, 1979; Sutcliffe, 1979). Such a comparison demonstrates that the *Hae*III fragment of ColE1, containing the origin of replication, appears to possess only a small number of base pair changes, whereas, the region of pBR322 corresponding to the *Hae*IIA fragment of ColE1, possesses a number of sequence differences that could greatly affect functions encoded within this region. In addition, the region containing the unique *Eco*RI site of ColE1 is absent from pBR322.

d) Plasmid recombination

The *Hae*IIB segment of ColE1 has been shown to contain a fragment 380bp long encoding a sequence designated *cer* (Fig.1.3), that is involved in site-specific recombinational resolution of plasmid multimers (Summers &

Sherratt, 1984). The cer recombination system is independent of host recA, recF and recE functions, and appears to be involved only in intraplasmidic recombination. It is not known whether the cer locus encodes a polypeptide product which may act as a recombinase, however, it has been suggested that a host encoded protein(s), may function as the recombinase.

1.2.2 Plasmid pBR322

a) Introduction

pBR322 is a widely used 4.36kb, ColE1-related cloning vector, constructed largely in vitro (Fig.1.10B), (Bolivar *et al.*, 1977). The origins of replication and transfer were derived from plasmid pMB1, whilst the ampicillin and tetracycline resistance determinants were generated from plasmids RSF2124 and pSC101 respectively (Fig.1.10A). The complete nucleotide sequence of this plasmid has been determined (Sutcliffe, 1979).

b) Plasmid copy number and stability

The copy number of pBR322 is slightly greater than that of ColE1, which presumably reflects the slight difference in copy number control that exists between plasmids ColE1 and pMB1 (Davison, 1984). However, both plasmids appear to be susceptible to copy number variation during batch culture growth. The copy numbers of plasmids ColE1 and pBR322 increase 5-fold and 4-fold respectively on transition from exponential to stationary phases of growth (Barry Polisky, personal communication, Indiana University, Bloomington, Indiana. 1982; Stueber & Bujard,

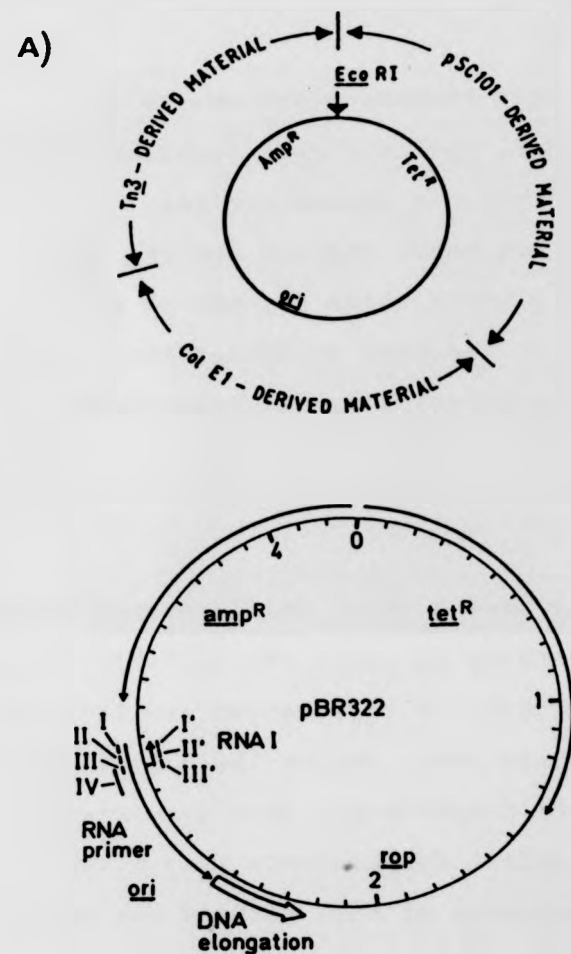
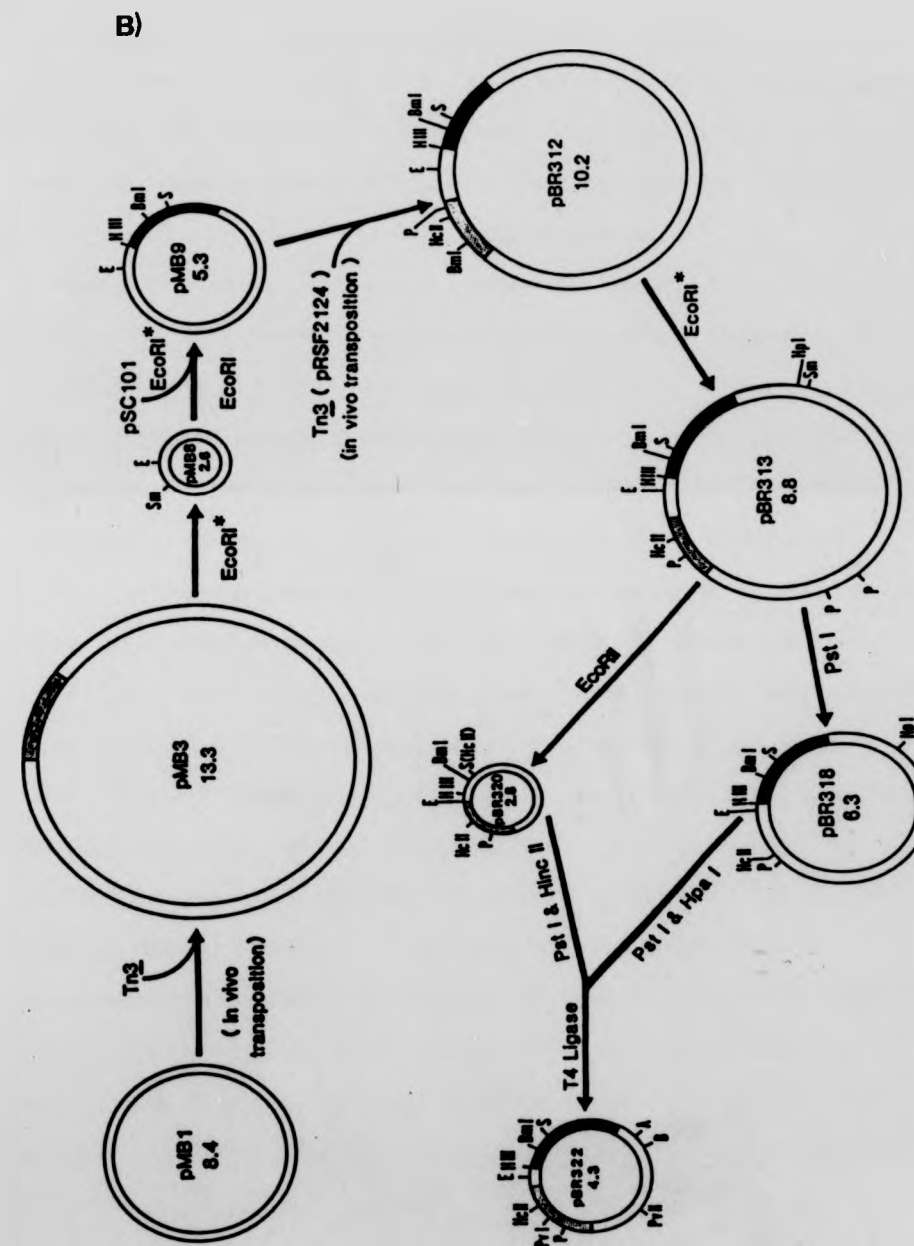


Figure 1.10

- A) The origin and structure of plasmid pBR322.
(Reproduced from (upper figure), Primrose et al., 1984 (lower figure), Davison, 1984).
- B) Diagrammatic representation of stages in the construction of plasmid pBR322 from plasmids pMB1, pSC101 and RSF2124. Plasmid sizes are given in kilobase pairs A, AvaI; B, BalI; BmI, BamHI; E, EcoRI; HIII, HindIII; HcII, HincII; HpI, HpaI; P, PstI; PvI, PvuI; PvII, PvuII; S, SalI; Sm, SmaI.
(Adapted from Bolivar, 1979).



1982). For pBR322, it has been concluded that the increase in plasmid concentration appears to correlate inverseley with the growth-rate of the culture (Stueber & Bujard, 1982). In addition, stationary phase cells were also observed to exhibit enhanced plasmid multimer formation, which could be abolished by the introduction of a recA mutation into the host background.

Plasmid pBR322 has been shown to be segregationally unstable during phosphate-limited chemostat culture in both recA⁺ (Fig.1.8), and recA E.coli K-12 strains (Jones et al., 1980b; Jones & Melling, 1984). Prior to the initiation of nutrient media-flow, a chemostat culture commences with batch culture growth in the chemostat vessel. Plasmid pBR322 may therefore have a variable copy number dependent on batch culture growth phase at the initiation of nutrient media-flow, which would undoubtedly affect the resultant kinetics of persistence of this plasmid during chemostat culture. The ColE1 derivative, pDS1109, has been shown to exhibit a 5-fold reduction in plasmid DNA content during chemostat culture (Fig.1.7B), (Jones et al., 1980b). This may now be cautiously interpreted as being a reversal of the 5-fold increase in plasmid copy number, that would have occurred during growth to stationary phase, before commencement of the chemostat experiment. A similar reduction in the plasmid DNA content of pBR322-bearing cells was likewise assumed to occur prior to the detection of plasmid-free segregants (Jones et al., 1980b). In addition, the kinetics of segregation of pBR322-free cells during phosphate-limited chemostat culture, has been shown to vary with both the

dilution rate and temperature (Wouters et al., 1980). A decrease in dilution rate, or an increase in temperature, was observed to result in the earlier appearance of plasmid-free cells. It was concluded that variations in the dilution rate and temperature altered the relative fitness of plasmid-bearing and plasmid-free cells, plasmid-free cells having a selective advantage over plasmid-bearing cells. Therefore, the relative fitness of pBR322-free cells increases with a decreasing dilution rate and increasing temperature. The segregation of plasmid-free cells suggests that a process of random distribution, rather than active partition, occurs for pBR322 and that daughter cells acquiring a low copy number may be expected to have a selective growth advantage over cells with a higher copy number.

Differences in the affinity for a limiting substrate have been suggested as being a decisive factor in the outcome of competition experiments (Wouter, et al., 1980), and it may be reasonable to assume that changes in fitness with respect to dilution rate and temperature, may reflect differences in plasmid copy number, having a direct bearing on the affinity of each cell for a limiting substrate. Indeed, it is notable that Wouters et al., (1980), observed a reduction in the activity of beta-lactamase before the appearance of plasmid-free cells, suggesting that a reduction in plasmid copy number may have occurred.

Host background can also be an important factor with respect to the stability of plasmid pBR322, as suggested by Noak et al., (1981). They have shown that pBR322 can be

stably maintained during glucose-limited chemostat culture at a dilution rate of 0.15 hr^{-1} and a temperature of 37°C , for at least 100 generations in E.coli strain GY2354, and for at least 75 generations in E.coli strain GM31. However, they did not rule out the possibility that their 'pBR322', may be different from that used in other chemostat studies (Jones et al., 1980b; Jones & Melling, 1984; Wouters et al., 1980; Vernet et al., 1985). In addition, the pBR322 deletion derivative pAT153 (Fig.1.11), (Twigg & Sherratt, 1980), has also been shown to exhibit differences in stability during chemostat culture with respect to host background, being stably maintained in E.coli strain HB101, but segregating plasmid-free cells in E.coli strain W5445 recA⁺. In contrast, plasmid pBR322 segregated plasmid-free cells in both these strains under the same conditions (Jones & Melling, 1984).

Segregational stability of pAT153 and pBR322 in E.coli strains such as HB101, GY2354 and GM31, may be due to the maintenance of a sufficiently high copy number, possibly as the consequence of a reduction in the transcription of plasmid-encoded functions, or an increase in the efficiency of any transcriptional terminators from which readthrough may interfere with plasmid replication (Stueber and Bujard, 1982). Indeed, it is notable that plasmids pBR327 and pBR328 are considerably more unstable than the parent plasmid pBR322 (Jones & Melling, 1984). pBR327 and pBR328 both contain deletions that bring the C-terminal coding region of the tetracycline resistance gene into close proximity to the origin of plasmid

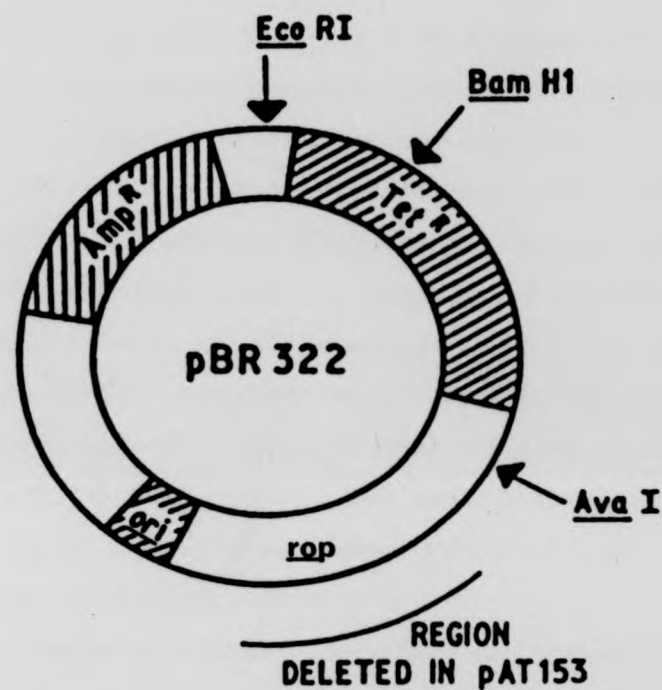


Figure 1.11

The structure of plasmid pBR322, and its HaeIII and H deletion derivative pAT153.

replication (Covarrubias et al., 1981). It has been shown that transcriptional readthrough from the tetracycline resistance region, under the control of the bacteriophage T5 promoter, P₂₀₇, towards the promoter which primes DNA replication can directly interfere with plasmid replication, in addition to indirectly causing the over production of the Rop protein (Stueber and Bujard, 1982). pAT153 contains a 0.62kb deletion that is overlapped by a 1.09kb deletion present within plasmids pBR327 and pBR328. It has therefore been suggested that the deletion in pAT153 may bring together adjacent sequences which are present but separated in pBR322, and absent altogether in pBR327 and pBR328, with a consequent influence on stability (Jones & Melling, 1984).

Finally, pBR327 is stably maintained following insertion of the par segment, from plasmid pSC101, into the region between the C-terminus of the tetracycline resistance gene and the replication origin (Zurita et al., 1984). It is notable that the par locus contains a putative transcriptional termination site, postulated to have some involvement in the partition process (Miller et al., 1983).

1.2.3 Plasmid CloDF13

a) Introduction

Cloacinogenic plasmid CloDF13 is a naturally occurring plasmid of Enterobacter cloacae. It is about 9.6kb in size, exists at a copy number of about 10 in E.coli K-12, and encodes the 59kDa bacteriocin cloacin

DF13 (Fig.1.12), (Hakkaart et al., 1981; van den Elzen et al., 1983). The replication and bom regions of CloDF13 show extensive similarities with the same regions of ColE1, both functionally, and as determined by nucleotide sequence analysis (Fig.1.13), (Veltkamp & Stuitje, 1981). However, CloDF13 is compatible with ColE1 and in contrast to ColE1 does not appear to form a relaxation complex (Veltkamp & Stuitje, 1981; Snijders et al., 1983).

b) Plasmid recombination and stability

There are two loci on CloDF13 designated parA and parB, deletion of either in a CloDF13 copy number mutant results in multimerization of the plasmid and plasmid loss from a minicell host background (Hakkaart et al., 1982). In contrast, the deletion of either parA and/or parB in a CloDF13 plasmid exhibiting a wild-type copy number, does not lead to loss of the plasmid. Increased expression of the RecE pathway (encoded by the Rac prophage in a recBC sbcA host), or the presence on the unstable plasmid of transposon-derived sequences encoding the Tn901 site-specific co-integrate resolution system, has been shown to restore stability as a consequence of reduced plasmid multimerization (Hakkaart et al., 1982).

Evidence suggests that the parB locus is a 328bp noncoding region that promotes recA-independent site-specific recombination in cis, utilizing host-encoded protein(s) to catalyse an intramolecular recombination event (Hakkaart et al., 1984). It is assumed that the host-encoded recombination system is only able to resolve a limited number of plasmid multimers, hence an increase

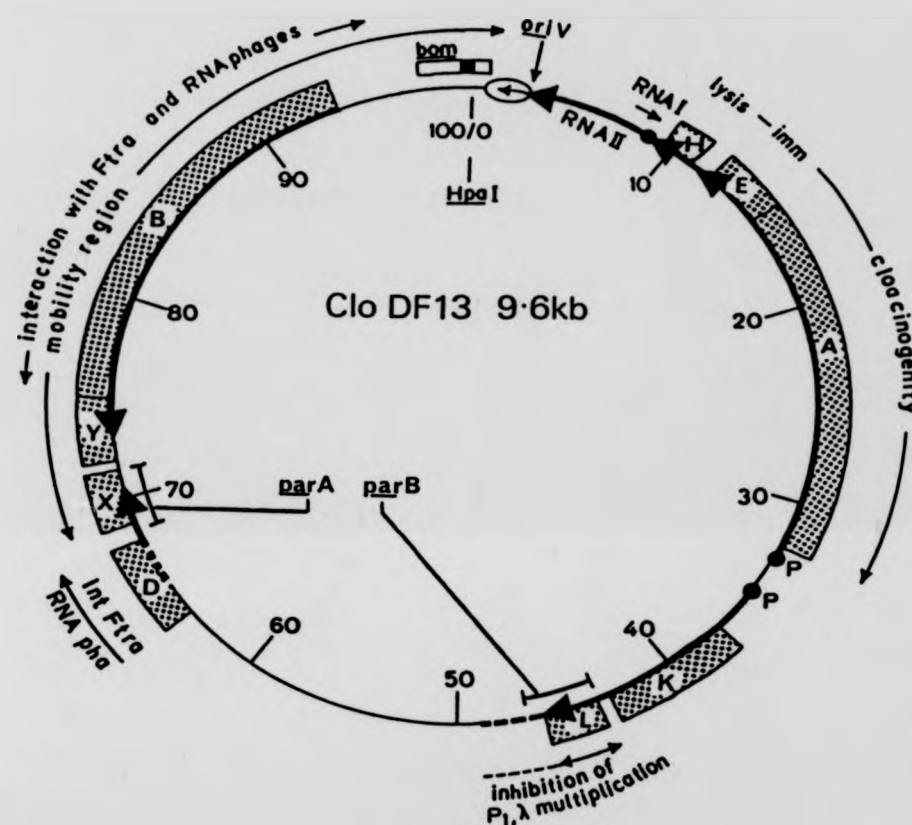


Figure 1.12

Genetic and functional map of plasmid CloDF13. The single HpaI site provides a reference point on the CloDF13 map. The plasmid genome can be conveniently divided into at least 4 functional regions, a) initiation and regulation of DNA replication (1.8% to 9%), b) bacteriocinogenity, immunity, lysis and transport of the bacteriocin (9% to 32%), c) plasmid maintenance (33% to 50%), and d) mobilization (67% to 1%).

(Reproduced from Snijders et al., 1983).

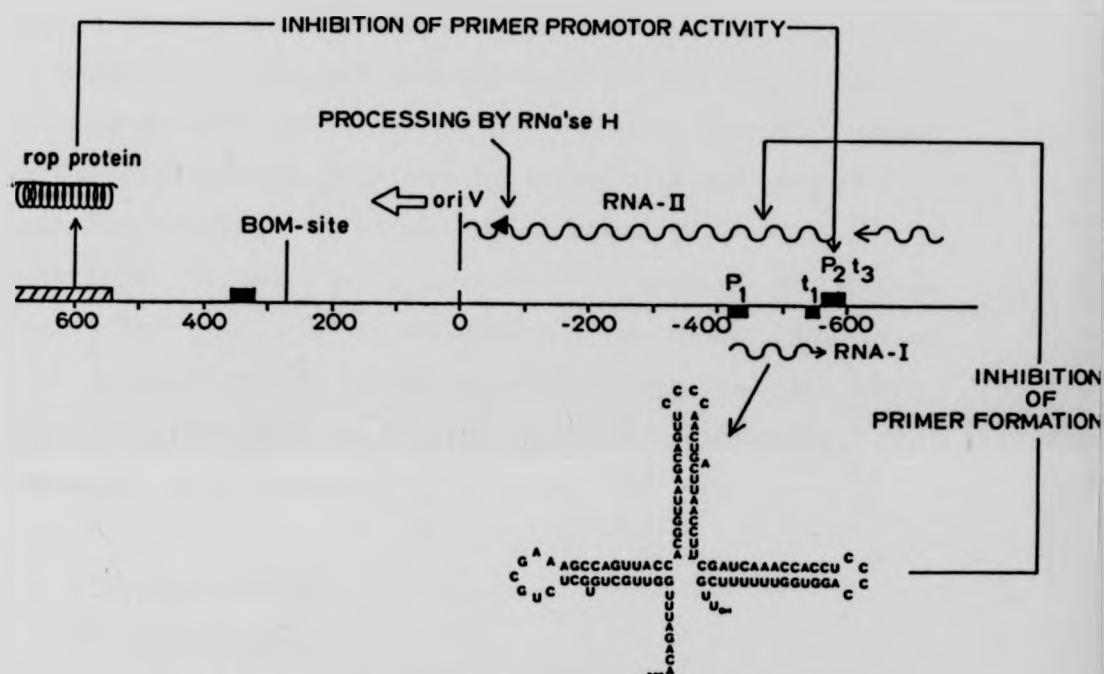


Figure 1.13

Schematic representation of the control of initiation of CloDF13 plasmid DNA replication.

(Reproduced from 'Replication of Prokaryotic DNA', book of abstracts, EMBO Workshop, May 1982, De Biehof, The Netherlands).

in plasmid copy number results in increased multimer formation (Hakkaart *et al.*, 1982). The function of the parA locus on the other hand, is uncertain, as it appears to make only a minimal contribution to the stability of plasmid CloDF13 (Hakkaart *et al.*, 1984). However, recent evidence has shown that the parA region possesses DNA sequence homology with the region specifying RNAI. Therefore, it has been suggested that RNAI or the region coding for RNAI, may in addition to functions associated with control of initiation of plasmid replication, also have an involvement in CloDF13 plasmid segregation (Hakkaart *et al.*, 1985).

1.2.4 Plasmid pSC101

a) Introduction

pSC101 is a naturally occurring plasmid of about 9.43kb, isolated from Salmonella panama, encoding tetracycline resistance. It has a copy number of 4-5, and is the only plasmid known to be absolutely dependent on the E.coli dnaA gene product for replication (Fig.1.14), (Armstrong *et al.*, 1984; Hasunuma & Sekiguchi, 1977).

b) Plasmid replication

Replication of pSC101 is unidirectional, initiating within a region encoding incompatibility and proceeding towards the adjacent par locus (Fig.1.15), (Churchward *et al.*, 1983). The structural features of this region include an A-T rich sequence, from which it is assumed DNA synthesis commences, and an adjacent but upstream region containing three direct repeats. This is an arrangement

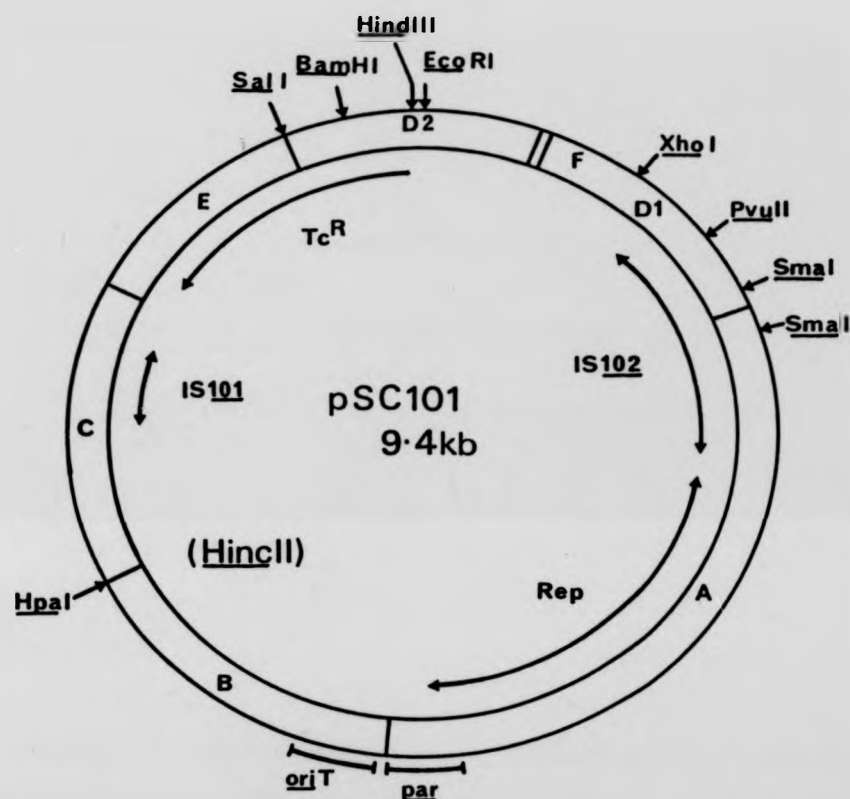


Figure 1.14

HincII genetic and functional map of plasmid pSC101. Indicating the location of the replication (Rep), partition (par), and transfer (oriT) regions, together with the tetracycline resistance (TcR), coding region, and the positions of insertion sequences IS101 and IS102. (Reproduced from Stoker et al., 1982).

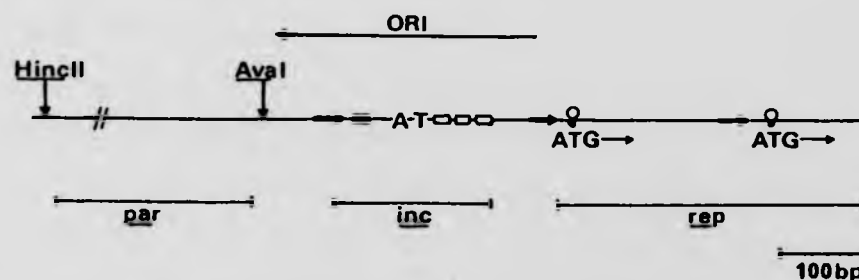




Figure 1.15

Diagrammatic representation of the maintenance region of plasmid pSC101. The heavy arrows indicate promoter sequences known to function in vivo. The location of two translation initiation signals (ATG), within the rep101 (rep), coding region are also shown. The A-T rich region and adjacent direct repeat sequences (), are also indicated, as is a sequence (), which is found repeated four times in the oriC region of the E.coli genome, and may function in binding the dnaA gene product. Replication initiates in the region marked ORI, and proceeds as indicated by the arrow. The locations of the incompatibility (inc), and partition (par), regions are also shown. (Reproduced from Churchward et al., 1983).

similar to, but showing no homology with other origins of plasmid replication, such as those of R6K, RK2, F and P1 (the number of direct repeats varies from three in pSC101 to nine in P1). It has been suggested that these direct repeat sequences function to negatively regulate the initiation of plasmid replication, by titrating an autoregulated initiation protein which is positively required for the initiation of plasmid replication (Chattoraj et al., 1985). In addition, there are two inverted repeats further upstream from the origin of replication each lying close to an upstream transcriptional promoter (Churchward et al., 1983; Armstrong et al., 1984). A 37.5kDa polypeptide is expressed from one of these promoters, the 316 amino acid Repl01 protein. The second promoter lies within the repl01 coding sequence and may lead to the production of a truncated protein (Armstrong et al., 1984). The Repl01 protein is able to specifically bind to the replication origin within the region containing the three direct repeats. It has been concluded that Repl01 functions as an origin recognition protein, which may interact with the host DnaA protein, guiding the replisome to the origin of plasmid replication within the A-T rich region. Binding of the Repl01 protein to the upstream inverted repeat region results in autorepression of repl01 transcription (Vocke & Bastia, 1985).

c) Plasmid partitioning

The par locus of pSC101 is located adjacent to, but functions independently of, the replication origin

(Meacock & Cohen, 1980). It acts in cis, suggesting that it does not encode a freely diffusible gene product but functions as a specific plasmid DNA site. It has been localized to a 270bp region, which does not specify plasmid incompatibility (Nordstrom et al., 1980b), and is able to function when its orientation is reversed, or when located elsewhere on pSC101, or in other unrelated replicons. The location of the par locus, close to the replication region of pSC101, may be the consequence of evolutionary processes that link such plasmid functions as replication and partition. However, activation of the partition mechanism, may in addition, be dependent on duplication of the par locus at either initiation or termination of plasmid replication. Indeed, it has been concluded from attempts to stabilize tryptophan (trp)-operon-bearing plasmids with the par locus (Skogman et al., 1983), that the observed remaining partial plasmid instability, is probably the result of transcriptional interference leading to a decrease in the full efficiency of the partition mechanism. The original instability of these trp plasmids (pBR322::trp and pACYC184::trp), has been attributed to a reduction in plasmid copy number, as a result of transcriptional readthrough into the replication region.

Sequence analysis of the par region reveals that it contains a rho-independent transcription terminator. It may be that this termination signal functions to block transcription through into a proximal DNA segment, having a particular role in the partition function (Fig.1.16), (Miller et al., 1983). Indeed, deletions containing the

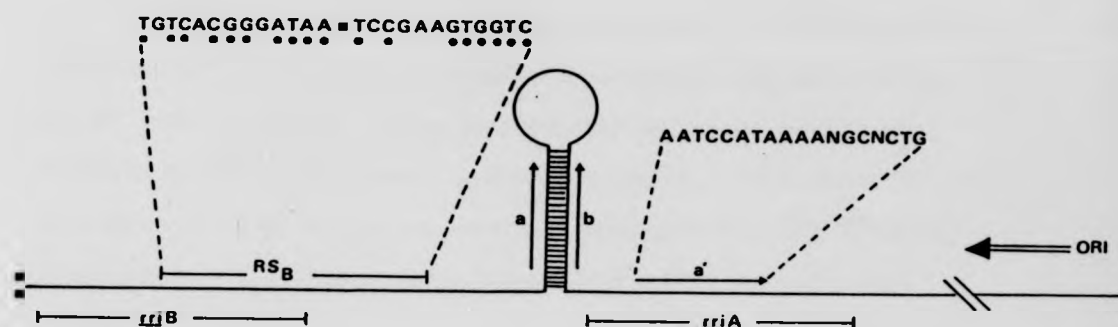


Figure 1.16

Structural features of the *par* region of plasmid pSC101, derived from DNA sequence analysis. A prominent feature of the *par* region is the presence of a *rho*-independent transcription terminator (stem and loop structure). One of the inverted repeat sequences contained within the stem region of this transcription terminator, occurs as a direct repeat further upstream towards the origin, and is represented by a single line arrow (the nucleotide consensus for this sequence is illustrated above the arrow). In addition to repeat sequences, the *par* region also contains sequences having homology with the *rrlA* and *rrlB* sites, occurring in the replication regions of plasmids ColE1 and pBR322, and the *RS_B* sequence, which promotes the formation of co-integrates and occurs in several plasmids. The extent of homology between the *RS_B* sequence and the *par* region, is indicated by dots under the relevant nucleotide sequence within the *par* region.

transcription terminator result in defective partition. The introduction of the bacteriophage fd transcriptional terminator, is however, unable to restore partitioning, which indicates that the region may have an additional function associated with partitioning. It is notable that a region to the right of the rho-independent terminator possesses significant homology with one limb of the cruciform structure of the transcription terminator, and that deletion within this region also demonstrates its importance to the functioning of par.

Structural and functional analysis of the par region of pSC101 has revealed a number of curious phenotypes associated with three partition-related (PR), segments that occur within a 100bp sequence (Tucker *et al.*, 1984). Two of these segments (a) and (b), exist as inverted repeat sequences that form the rho-independent terminator. The third PR segment (a'), occurs as a direct repeat sequence of (a), and is located to the right of the rho-independent terminator (Fig.1.16). Deletion of all three PR segments results in a plasmid which exhibits a super par⁻ phenotype, that is a random plasmid segregation rate consistent with a copy number much lower than is the actual case. In contrast, deletion of the (a) or (a') segment does not result in a par⁻ phenotype. However, deletion of (a) and (a'), or (a) and (b), or (b) and (a'), does give rise to a par⁻ phenotype, but with a rate of random plasmid segregation consistent with copy number (i.e., not a super par⁻ phenotype). Interestingly, both the par⁻ and par⁺ deletion mutants exhibit a reduced ability to co-exist with a wild-type pSC101 par plasmid.

This phenotype, designated cmp (competition), appears to be associated with the replication and incompatibility system of plasmid pSC101, since a pSC101cmp plasmid can readily co-exist with a pACYC184 recombinant plasmid carrying a wild-type pSC101 par region. It would appear, therefore, that mutations which may occur within the par region of wild-type pSC101 plasmids, may be positively selected against by the remaining wild-type par plasmids within a host cell.

It has been suggested that the rho-independent terminator region may function as a protein-binding or membrane-binding site. Indeed, it has been shown that there is a strong correlation between plasmid replicons carrying the par locus of pSC101, and their ability to bind to membrane material of about the same density as that of the outer membrane fraction of E.coli K-12 (Gustafsson *et al.*, 1983). Furthermore, the rho-independent terminator, may in addition, aid plasmid stability by preventing transcriptional readthrough interference during initiation of plasmid replication, when appropriately positioned with respect to the origin of plasmid replication (Stueber & Bujard, 1982).

The nucleotide sequence of the par region also shows homology with the rriA and rriB sites of ColE1 and pBR322 (Miller *et al.*, 1983). These sites interact with the E.coli replication factor, factor Y, thought to initiate DNA synthesis on single-stranded DNA templates (Soeller & Marians, 1982). The rriA site, located on the H-strand, is thought to be involved in second-strand synthesis. While the rriB site, present on the L-strand, is thought

to be involved in the conversion of single-stranded DNA to duplex DNA following plasmid transfer, either by conjugation or mobilization. However, the oriT site of pSC101, located adjacent to but outside the par region, suggests that the par region may not be directly involved in pSC101 mobilization (Fig.1.14).

The par region of pSC101 is unable to stabilize oriC plasmids completely (Hinchliffe *et al.*, 1983). Essentially, oriC plasmids consist of selectable nonreplicating fragments of plasmid DNA in which a plasmid replicon is replaced by the oriC region of the E.coli chromosome. Such plasmids exhibit marked instability, either as a consequence of being defective in functions related to the segregation of the E.coli chromosome, or due to the presence of additional copies of oriC. This instability can be rectified by the addition of the par region from plasmid F, but not from pSC101 (Ogura & Hiraga, 1983a; Hinchliffe *et al.*, 1983). It has been suggested that, the inability of the pSC101 par locus to stabilize oriC plasmids, may be the consequence of the pSC101 partition mechanism being able only to stabilize heterologous replicons possessing a second cis-acting site, possibly located within the replication region. This site is presumed to be absent from the replication region of oriC plasmids (Hinchliffe *et al.*, 1983). The presence of the rriA site, located downstream from the origin of pSC101 replication but present within the essential sequences necessary to maintain the fidelity of plasmid segregation, may therefore have some significance

(Miller et al., 1983).

Finally, the sequence of the par region of pSC101 has also been shown to contain homology with sequences located on several plasmids that promote, via a sequence-specific recombination process, the formation of plasmid co-integrates in a Staphylococcus aureus strain. This sequence-specific recombination process is mediated either by host- or phage-encoded recombination functions (Novick et al., 1984). The existence of these sequences, termed RS_B in all of the six plasmids studied, and their homology with the par region of pSC101, suggests that they may have a role in addition to being recombinogenic sites, or, that homologous recombination may play a role in plasmid partitioning. The presumptive recombinogenic property of the par locus of pSC101 may therefore additionally contribute to an increased plasmid stability, either as a consequence of integration into the host chromosome, or resolution of plasmid multimers.

1.2.5 Plasmid R1

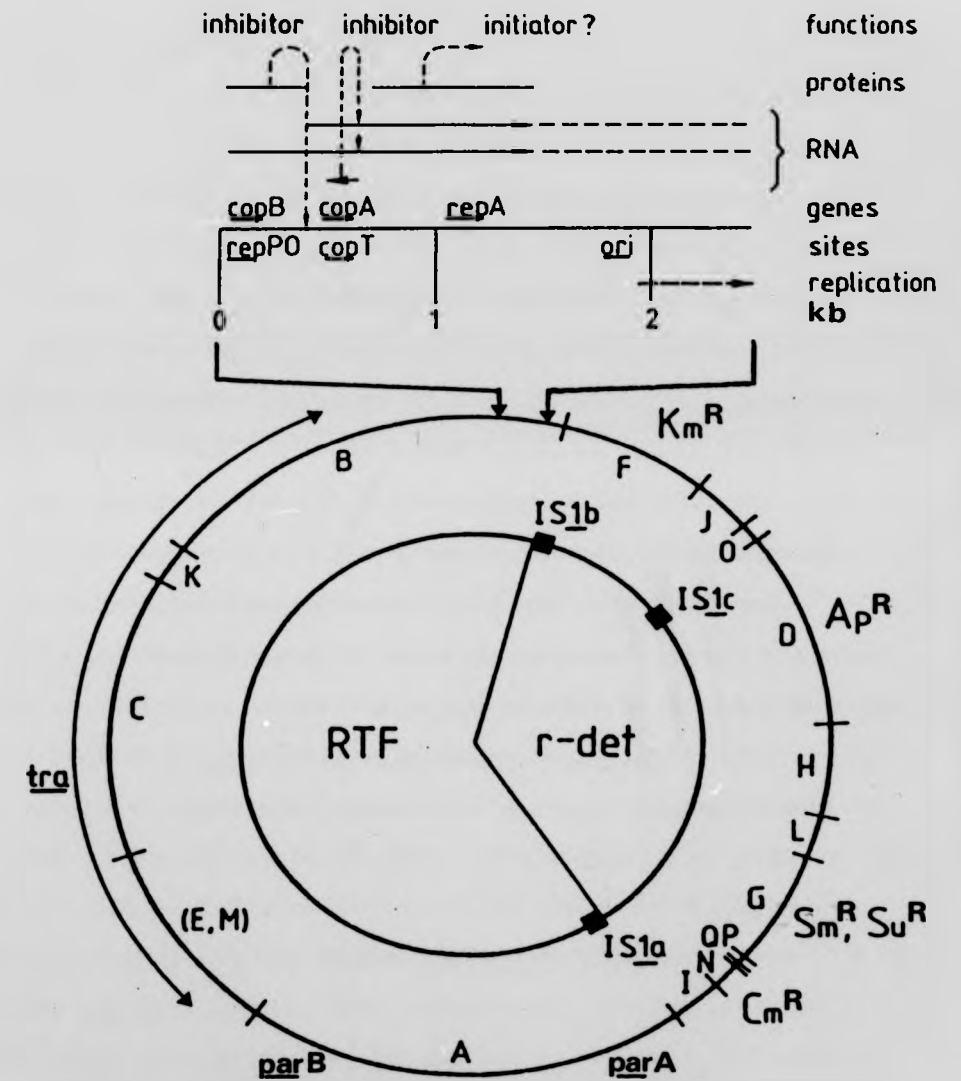
a) Introduction

R1 has a molecular weight of about 58MDa and exists at a copy number of about 2-3. It comprises of two distinct regions that are separated by insertion sequences, the resistance transfer factor (RTF), and the r-determinant (r-det.), (Fig.1.17). The latter region encodes five antibiotic resistance genes conferring resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and sulphonamides. The RTF region carries genes conferring conjugal plasmid transfer, together with the origin of

Figure 1.17

Genetic and functional map of plasmid R1.

A to Q refers to fragments generated by restriction endonuclease EcoRI. The r-determinant (r-det), is flanked by two IS1 sequences (insertion sequences), and another IS1 sequence occurs within the r-det itself. The r-det specifies resistance to five antibiotics; ampicillin, Ap^R; chloramphenicol, Cm^R; kanamycin, Km^R; streptomycin, Sm^R and sulphonamides, Su^R. The RTF region specifies functions which confer plasmid conjugal transfer (tra), replication, and partition (parA and parB). The basic replication region, is expanded to illustrate the functional components contained within this 2.5kb sequence. (Adapted from Nordstrom et al., 1984).



vegetative replication and partition functions (Nordstrom et al., 1984; Gerdes et al., 1985a)

b) Plasmid replication

Replication of R1 proceeds unidirectionally into the r-det., initiating in a 2.5kb region of DNA constituting the basic replicon. This minimal replicon contains four important elements related to plasmid replication control, three genes copA, copB and repA, together with an origin of replication (ori), (Fig.1.17), (Light & Molin, 1982). However, the exact location of the origin and the nature of the replication priming events are at present unknown.

repA appears to code for a polypeptide that is positively required for autonomous plasmid replication, and according to DNA sequence studies, the repA gene product may be expected to be a protein of some 278 amino acids, most likely functioning as a replication initiation factor (Ryder et al., 1982; Stougaard et al., 1981b). In contrast, the copA gene codes for a small unstable RNA of about 90 nucleotides in length. This small RNA acts as an inhibitor of R1 plasmid replication (Stougaard et al., 1981a). It has a high degree of secondary structure (Gerhart et al., 1986), and mutational studies strongly suggest that the copA-RNA functions to inhibit plasmid replication, via a nucleic acid-nucleic acid interaction. Indeed, copA mutants that have lost all copA activity against wild-type plasmid R1, still retain copA activity against themselves, suggesting that a mutation in the effector coding sequences also leads to a similar change in the target sequences (i.e., copT), (Fig.1.17),

(Stougaard *et al.*, 1981a; Light & Molin, 1982). A second inhibitor of plasmid R1 replication is encoded by the copB gene. This gene expresses a protein of 86 amino acids that does not affect R1 incompatibility (Riise *et al.*, 1982). The two control genes, copA and copB, appear to act synergistically since double mutants, copA copB, are lethal to the host bacterium as a result of uncontrolled plasmid replication, so-called 'run-away' replication (Riise *et al.*, 1982; Stougaard *et al.*, 1981b).

Negative control of R1 replication is accomplished by the products of the copA and copB genes, they do so by negatively controlling the expression of the repA gene, whose protein is required positively for the initiation of plasmid R1 replication (Light & Molin, 1982). Each round of plasmid R1 replication requires *de novo* synthesis of the RepA protein, suggesting that it may be unstable or consumed during or following initiation of plasmid replication (Diaz & Ortega, 1984). The copA and copB gene products have distinct targets at which they exert their control (Fig.1.17), (Light & Molin, 1982; Nordstrom *et al.*, 1984) . The target for the CopB protein is the operator repO, and binding causes the inhibition of repA-RNA transcription from repP. However, the CopB protein does not appear to affect transcription from the upstream copB promoter, enabling this transcript to pass through the repO region. This copB extended transcript and the transcript initiating at repP both include the repA coding sequence, but nothing is at present known concerning the endpoints for these two transcripts, or their possible use as primers of DNA synthesis at the

origin of replication. copA-RNA, which is transcribed away from the origin of replication, appears not to affect the transcription of either copB or repA, but instead acts at a post-transcriptional level, either inhibiting translation of repA-mRNA, or possibly affecting primer formation at the origin, or both (Light & Molin, 1982). Quantitatively, the control of plasmid R1 replication may be initially regarded as the ratio between functional copA-RNA inhibitor and its target copT, since the formation of both copA-RNA and copB protein is constitutive and therefore proportional to gene dosage.

c) Plasmid partitioning

Plasmid R1, although normally present at a low copy number, is nevertheless stably maintained. This suggests the presence of an R1-encoded partition mechanism that ensures the distribution of plasmid copies to daughter cells at division. Indeed, deletion derivatives of plasmid R1 that fail to be stably inherited, either lack all or a portion of the EcoRIA fragment present within the RTF region (Nordstrom *et al.*, 1980a). Studies have recently demonstrated the presence of two independent stability functions, designated parA and parB, that are encoded within the EcoRIA fragment (Fig.1.17), (Gerdes *et al.*, 1985a). The parB function is able to stabilize unrelated plasmid replicons more efficiently than is the parA function, which suggests that parA may require some other factor associated with plasmid R1 replication. Indeed, transposon insertion within a region outside of but very close to the replication origin of R1, leads to

the instability of an RlparA⁺ plasmid, lending support to this suggestion. Both parA and parB have been shown to exert incompatibility, but only against the homologous par region. In addition, the parB⁺ region in contrast to the parA⁺ region is also able to exert incompatibility against parA⁺ parB⁺ plasmids. Such differences between the two par regions with respect to plasmid stabilization and incompatibility, indicates that the mechanisms by which these par regions mediate the stable inheritance of plasmid R1 may be different. Recent evidence suggests that parB may function to couple plasmid replication to the host cell cycle (Gerdes et al., 1985b). Finally, the absence of incompatibility associated with the par region of pSC101, contrasts with the incompatibility shown by the R1 par functions, even though both pSC101 and R1 par regions have been shown to specifically associate with membrane fractions of E.coli K-12 (Nordstrom et al., 1980b; Gustafsson et al., 1983).

1.2.6 The F plasmid and bacteriophage P1

a) Introduction

F is a 94.5kb conjugative plasmid existing at a copy number of one (unit copy) (Seelke et al., 1982; Lane, 1981). Likewise, bacteriophage P1 which in the prophage state is maintained as a plasmid of about 90kb, also exists at a copy number of one (Sternberg et al., 1981). Despite the unit copy number of F and prophage P1, they nevertheless segregate with a high degree of fidelity to daughter cells at cell division. Usually fewer than one in 10⁴ cell divisions gives rise to a prophage P1 free

progeny (Abeles et al., 1984). Therefore, the co-ordination of plasmid replication and partition in these plasmids must be tightly controlled with respect to cell division.

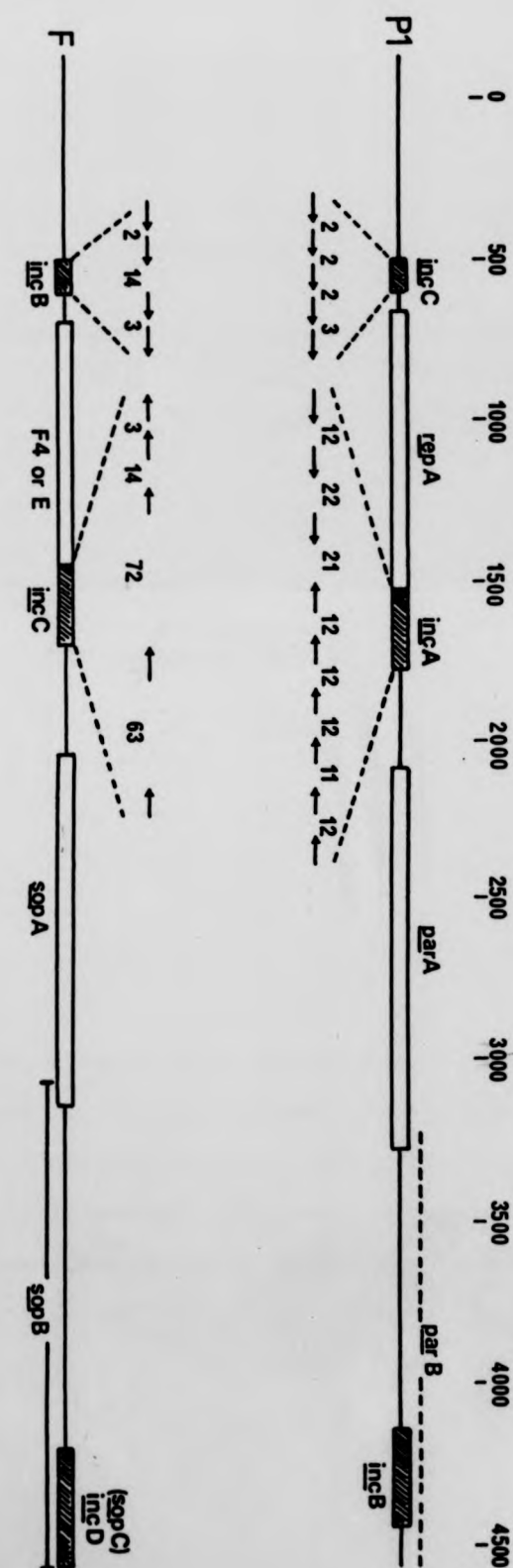
b) Plasmid maintenance and incompatibility

Both F and P1 have maintenance regions of a similar size (about 4-5kb). These regions, although not homologous, nevertheless have similar structural and organizational features, each replicon having similarly located areas encoding functions related to replication and partitioning (Fig.1.18), (Abeles et al., 1984). In addition, F has also been shown to encode a region whose function is responsible for the coupling of plasmid replication to host cell division (Fig.1.19) (Miki et al., 1984a).

Each maintenance region has been shown to contain three incompatibility loci. Two are associated with DNA replication, and have been designated incC and incA in prophage P1, and incB and incC respectively in the F plasmid. These loci, in both F and P1, flank an open reading frame that is presumed to encode the Rep protein specific to each replicon (Fig.1.18). The third locus, designated incB in prophage P1 and incD in the F plasmid, resides within the par region. These par loci have been shown to act in cis with polypeptides encoded by their respective plasmids. Either a single P1, or one of the two Par proteins of F, bind to their respective par loci, via a protein-DNA interaction that presumably catalyses the segregation of progeny replicons (Austin & Abeles, 1983a & b; Ogura & Hiraga, 1983a). These Par proteins

Figure 1.18

Structural comparison of the maintenance regions of the F plasmid and bacteriophage P1. The size of the maintenance regions are indicated in base pairs at the top of the diagram, as is the spacing between the 19bp repeat sequences occurring in each of the incompatibility determinants of the replication regions (i.e., *incC* and *incA* in P1, and *incB* and *incC* in F). The location and orientation of these repeat sequences are indicated by arrows. Sequences encoding proteins required for plasmid maintenance are indicated by open bars, except the *sopB* region. The location of a possible additional P1 partition protein is indicated by a broken line (Abeles *et al.*, 1985). Regions exerting incompatibility are shown as shaded bars. (Reproduced from Abeles *et al.*, 1984).



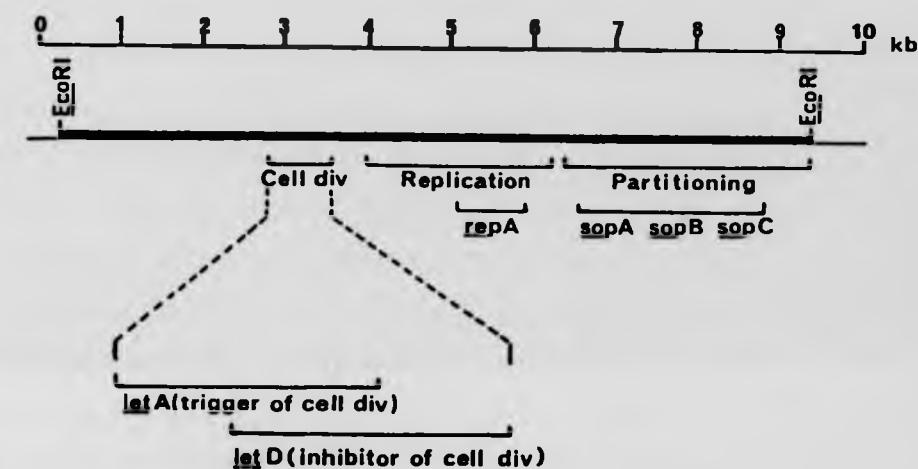


Figure 1.19

Organization of the maintenance region of the F plasmid. Regions required for the coupling of cell division with plasmid DNA replication (cell div), for replication and for plasmid partitioning are indicated.

(Adapted from Miki *et al.*, 1984b).

are encoded within sequences that are located between the cis-acting loci and the replication region (Fig.1.18).

The replication regions define the primary replication sites at which the majority of plasmid DNA replication events are initiated, presumably leading to bidirectional replication in a similar manner to that of the E.coli chromosome. However, both F and P1 also encode secondary replication regions (Seelke et al., 1982; Gardner et al., 1985; Sternberg & Austin, 1983). That for F has been localized to a region left of the primary replicon. This secondary replication site, however, is dependent on functional E.coli DNA polymerase I, but unlike ColEI-related plasmids, does not permit amplification in the presence of chloramphenicol (Lane, 1981). P1 has likewise been shown to contain a secondary replication site, also adjacent and to the left of the primary replication region. Evidence suggests that P1 also encodes a repressor of the secondary replication function, however, this repressor is not encoded within the region encompassing both the primary and secondary replication sites (Sternberg & Austin, 1983). Both F and P1 are segregationally unstable when replication is directed by the secondary replication functions. The possession of a secondary replication system, which is inherently inefficient, may act as a 'back-up' when the primary system normally governing replication is inactivated, therefore, partially ensuring plasmid maintenance (Lane, 1981; Sternberg & Austin, 1983).

Unfortunately, little is known concerning the actual mechanisms by which F or P1 initiate DNA replication,

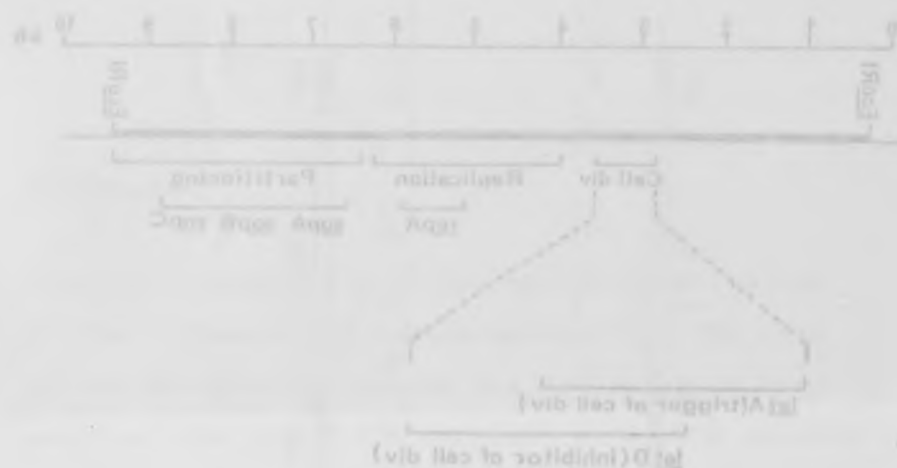


Figure 1.18

Organization of the maintenance region of the F plasmid. Regions required for the coupling of cell division with plasmid DNA replication (cell div), for replication and for plasmid partitioning are indicated. (Adapted from Miki et al., 1984b).

beyond the fact that such an initiation event appears to be negatively controlled and does not require the *E.coli* dnaA gene product. However, a sequence postulated to be the binding site for this protein, which is essential for replication of the *E.coli* chromosome, is present on both plasmids (Abeles *et al.*, 1984). Furthermore, the incA region of P1 has been shown to be capable of switching off P1 DNA replication in-trans. This may indicate that either incB or incC or both determinants on plasmid F, may also play an analogous role to that of incA in prophage P1 (Fig.1.18), (Austin & Abeles, 1983b).

c) Plasmid partitioning

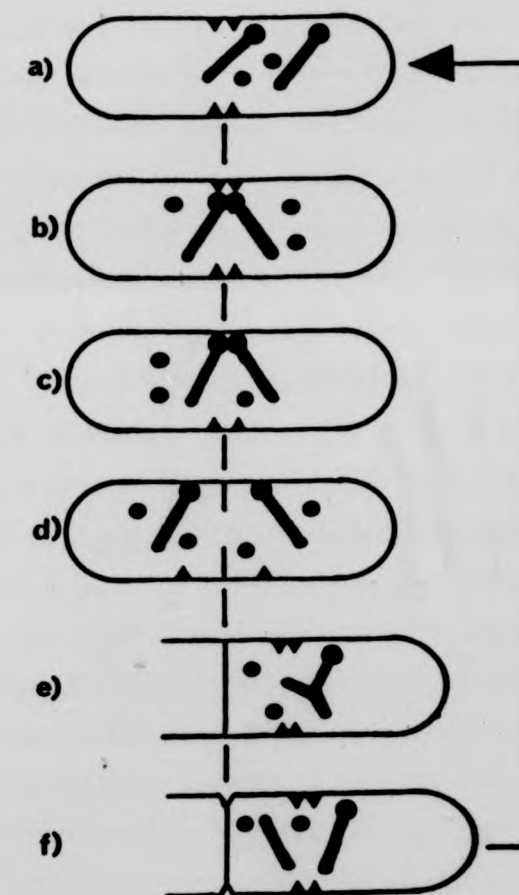
Both F and P1, have par regions adjacent to but functionally distinct from their origins of replication. The par region of P1 consists of about 2.5kb, and encodes a 44kDa protein that appears to act in-trans, by what is thought to be a specific binding to the adjacent incB locus (Fig.1.18), (Austin, 1984). A model that has been proposed to account for the interaction between the P1 par factors, assumes that binding of a monomeric Par protein to the incB locus, forms a complex which when 'active' allows the DNA-bound Par protein to form a dimer with a similarly bound P1 Par protein resulting in the pair formation of daughter plasmid copies. These DNA-bound dimer complexes are now presumed to associate or bind with a host factor(s), that defines the cell division plane. Septum formation at this plane, leads to the partitioning of daughter plasmids either side of the division septum (Fig.1.20), (Austin & Abeles, 1983b).

Figure 1.20

Schematic representation of a model for the partition of unit-copy plasmids.

- a) Monomeric Par proteins (dots), possess DNA-binding activity. They specifically bind to the inc site within the par region.
- b) Only DNA-bound Par proteins are capable of forming dimers. This results in pairing of the daughter plasmid copies.
- c) DNA-bound dimers are able to attach to any one of a number of identical binding sites, which define a future plane of cell division.
- d) Growth of the cell envelope, and subsequent septum formation, partitions the daughter plasmids as proposed by Jacob et al. (1963).
- e) Replication of plasmid DNA, triggers detachment of the complex from the cell envelope.
- f) Termination of plasmid replication allows the cycle to recommence.

(Reproduced from Austin & Abeles, 1983b).



In contrast, the par region of F is slightly larger than that of P1, consisting of a sequence of about 3kb (Fig.1.18). The par functions of F are encoded by two tandemly arranged genes, sopA and sopB, each having their own promoter and encoding 41kDa and 37kDa polypeptides respectively. Adjacent to the sopB coding region lies the sopC locus, a cis-acting site within which are contained 12 copies of a 43bp direct repeat (Helsberg & Eichenlaub, 1986). The sopB gene product is able to exert incompatibility, presumably by binding to the sopC locus. Indeed, attempts to clone segments containing the adjacent sopB sopC region, into the multicopy vector pBR322, have been unsuccessful (Ogura & Hiraga, 1983a). The majority of clones obtained possessed deletions of the sopC region. It was concluded that the sopC locus may only be cloned, on condition that the sopB coding region is absent. This would suggest that a strong interaction may occur between the SopB protein and sopC locus, presumably leading to inhibition of pBR322 replication. However, cloning of the entire par region derived from F is possible, and leads to segregational stability of pBR322. In addition to the binding of the SopB protein, two other supposedly host-encoded proteins of about 33kDa and 75kDa were also reported to bind to the sopC region (Fig.1.21). The regulation of expression of sopA and sopB is as yet unknown, however, preliminary observations suggest that a SopA/SopB complex may function as a repressor of the sopA gene, while the sopA gene product may function to positively regulate the sopB gene (Fig.1.21), (Ogura & Hiraga, 1983a; Austin & Wierzbicki, 1983).

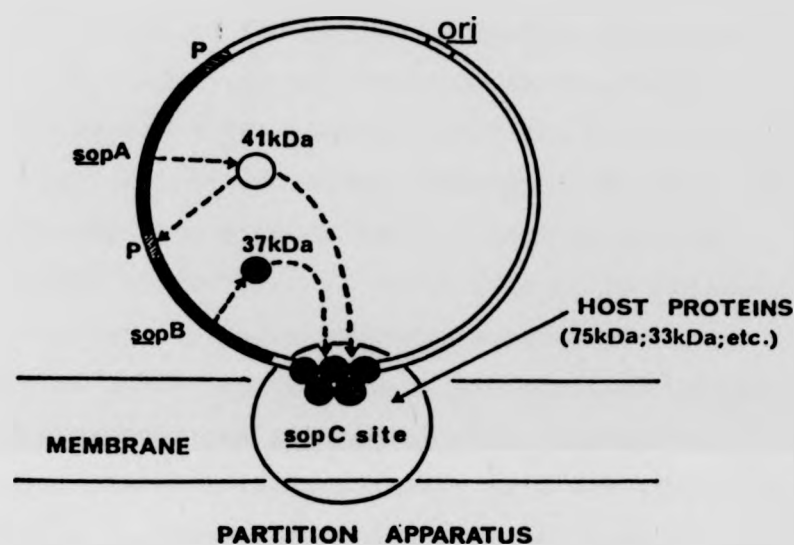


Figure 1.21

Diagrammatic representation of an hypothetical partition apparatus for the F plasmid. Interaction of both plasmid (SopA and SopB), and host-encoded proteins with the *sopC* locus, is presumed to form a partition complex at a site within the membrane. The expression of the *sopB* gene product is postulated to be positively regulated by the *sopA* gene product, while expression of *sopA* may be controlled by a SopA/SopB protein complex (not illustrated in diagram).

(Reproduced from Ogura & Hiraga, 1983a).

The model put forward to account for Pl partitioning may also apply to F, particularly with respect to the proposed interaction of the sopB gene product with the sopC locus (Fig.1.20). It is, however, interesting to note that the Pl and F maintenance functions do not exert incompatibility against each other (Austin & Abeles, 1983b), which may suggest that the cellular partition apparatus in the proposed model would have to be able to distinguish between Pl and F daughter molecules.

Finally, as mentioned previously, in contrast to the par region of pSC101, the par region of F is able to stabilize oriC plasmids (Hinchliffe *et al.*, 1983; Ogura & Hiraga, 1983a). Perhaps the involvement of plasmid-encoded Par proteins may be a specific feature only of unit-copy plasmids, in which replication control is similar to that of the host chromosome and where accuracy of partitioning is of course vital to maintenance.

c) Cell division coupling to plasmid replication

When F plasmid replication is blocked, the host cell divides once, division then ceases and the cells form nonseptate filaments (Miki *et al.*, 1984a). The single generation period, occurring prior to filamentation, is presumed to be a consequence of a reduction in the number of F copies to one per cell. Mutations within an adjacent region, located to the left of the replication region, appear to define two cistrons, letA and letD, which are responsible for the plasmid-mediated cell division inhibition (Fig.1.19). These let mutations, in an unsuppressed background, result in host cell death as a

consequence of cell division inhibition and filament formation. Furthermore, these mutations also lead to the induction of lambda prophages.

The F plasmid-encoded cell division control region appears to specify two gene products, the LetA (CcdA) and LetD (CcdB) polypeptides, that have molecular weights of about 8kDa and 11kDa respectively. These genes are contained within an operon, whose expression appears to be controlled by termination of plasmid DNA replication (Karoui et al., 1983). letA appears to code for a nondiffusible protein, since it is only able to act in cis, and is thought to function in a similar manner to the termination protein proposed to be involved in the coupling of host DNA replication to cell division (Fig.1.22), (Jones & Donachie, 1973). Furthermore, the cell division control region of F, has been shown to be functionally independent of both the rep and par regions, since it can be either translocated or inverted, and still express its cell division control functions (Miki et al., 1984a).

Such plasmid-encoded cell division control functions may be expected to exist, for unit-copy plasmids, whose replication is not synchronized with that of the host chromosome. Indeed, evidence has been presented indicating that F may replicate independently of the host cell cycle, thereby suggesting the necessity for plasmid controlled host cell division (Andresdottir & Masters, 1978). However, other studies have arrived at opposing conclusions, and it may be that F is capable of both synchronous and asynchronous replication with respect to

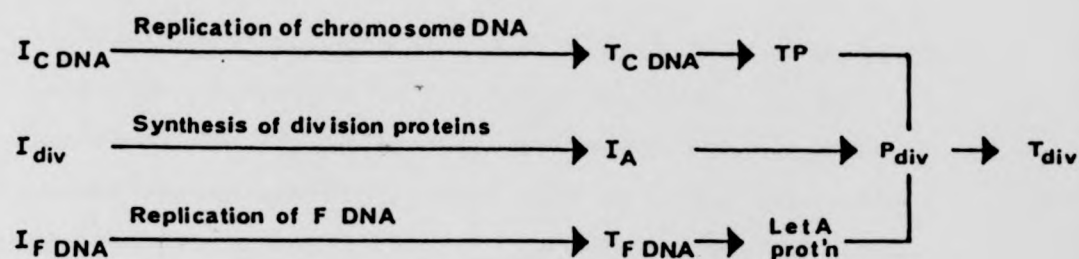


Figure 1.22

Diagram of a model to account for the coupling of cell division and DNA replication in *E. coli* cells bearing the F plasmid. The model represents a modification of that proposed by Jones & Donachie (1973), for the coupling of chromosomal replication to cell division. In this model initiation of chromosomal replication (I_C DNA), occurs when a cell attains a doubling in its initial mass. Initiation of F replication (I_F DNA), is assumed to occur at any point during the cell cycle. The termination of chromosome replication (T_C DNA), and F replication (T_F DNA), results in the synthesis of a termination protein(s) (TP), and LetA protein respectively. Initiation of assembly of the division septum occurs upon completion of the synthesis of division proteins. Interaction between the newly assembling septum, termination protein, and LetA protein (P_{div}), leads to cell division (T_{div}).

(Reproduced from Miki et al., 1984b)

the host chromosome (Lane, 1981).

Inhibition of cell division in E.coli appears to be under the control of at least two pathways, the sfi-dependent and sfi-independent pathways, which appear to couple chromosome replication with cell division (for a more detailed description of the SOS regulon and division inhibition see page 112, Section 1.3. 'Control of cell division in E.coli K-12'). The sfi-dependent pathway, a component of the SOS regulon, is presumed not to be involved in the regulation of cell division and chromosome segregation during normal growth, that appears to be the function of the sfi-independent pathway which at present is not fully understood (Burton & Holland, 1983).

F-encoded inhibition of cell division appears to be closely related to the sfi-dependent pathway, and therefore to the SOS regulon. Indeed, letA mutants are only capable of a recA-dependent induction of lambda prophages. However, the inhibition of cell division by letA mutations is not dependent on a recA gene product. The sfiA gene product, a component of the sfi-dependent pathway and a postulated host-encoded inhibitor of cell division, is however, synthesized in enhanced amounts in a recA-independent manner following its induction by the mini-F amber-mutation ham22. This mutation causes the virtual disappearance of a polypeptide believed to be the letA gene product. Therefore, it would appear that loss of the LetA function, leads to induction of the sfiA gene by an interaction independent of the normally required RecA protease activity, leading to subsequent inhibition of cell division (Karoui et al., 1983). However, recent

evidence seems to indicate that F plasmid-mediated inhibition of cell division is independent of both the sfiA and sfiC functions (Hiraga *et al.*, 1985), therefore, the mechanism by which F plasmid replication is coupled to host cell division is at present not clear.

An hypothesis accounting for F-encoded regulation of cell division has been proposed (Miki *et al.*, 1984b). This model suggests that host processes leading to cell division are coupled to the completion of F replication (Fig.1.22). Genes, letA and letD, responsible for this coupling, presumably code for polypeptides which are required for the control of cell division. LetD polypeptide, present from a previous round of plasmid replication, acts to prevent cell division. When F replication is completed, synthesis of letA and letD gene products takes place. letA gene product acts to suppress the activity of the LetD polypeptide, preparing the cell for the next division. This event will only take place when both chromosomal and plasmid replication is completed, and the postulated termination protein of the host chromosome, and the LetA polypeptide of the F plasmid are functionally present (Fig.1.22). LetA and functionally suppressed LetD polypeptides are presumably consumed or inactivated during the cell division process, resulting in activation of the nascent LetD polypeptide on distribution to each daughter cell during division, thus preventing subsequent division until the next round of plasmid replication is completed.

The involvement of plasmid-encoded functions in controlling the process of cell division is then perhaps a

specific feature of unit-copy plasmids, as plasmid-encoded Par proteins appear to be. Indeed, replication of Pl has been shown to be asynchronous with respect to either initiation or termination of chromosome replication (Prentki *et al.*, 1977), and Pl may therefore be expected to encode a similar system to that observed in F, of plasmid replication/cell division coupling, thus contributing to the maintenance of the Pl prophage genome.

e) Plasmid co-integrate resolution

Pl prophage genomes, deficient in a site-specific resolution system, tend to form dimers as a consequence of generalized recombination, thereby interfering with the normal partition process and giving rise to plasmid-free progeny (Fig.1.2), (Austin *et al.*, 1981). This site-specific recombination system encoded by plasmid Pl, consists of a cis-acting locus loxP, together with a specific recombinase encoded by cre, that catalyses site-specific recombination at the loxP site (Hoess & Abremski, 1984). The cre gene product, a 35kDa polypeptide, forms a dimer in the presence of $MgCl_2$, this dimer is capable of specifically binding to a single loxP site (Abremski & Hoess, 1984). The protein-DNA interaction protects about 34bp of DNA, which contains two Cre protein specific binding domains. Each domain comprise a 13bp inverted repeat, together with 4bp of adjacent DNA which contributes to an 8bp spacer region between the inverted repeats.

The loxP-cre system carries out two important

functions. The first accomplishes the circularization of linear P1 DNA upon entry into a host bacterium. This is conditional on the P1 phage genome containing two loxP sites, normally the result of terminal redundancy during packaging. The second is the site-specific resolution of P1 plasmid dimers to monomers (Abremski & Hoess, 1984). For the latter intraplasmidic recombination reaction, it appears that the efficiency with which recombination occurs is independent of both the orientation of the loxP sites, and as shown in vitro, the conformation of the P1 genome (i.e., supercoiled or linear). Notably, the Cre recombinase has only a requirement for Mg^{2+} , no external energy co-factors appear to be necessary. Other studies of the loxP-cre system have shown it to be capable of catalysing interplasmidic recombination, which occurs at a much lower frequency than intraplasmidic events. When the substrates for interplasmidic recombination are both supercoiled, the product is a supercoiled dimer, but when one substrate is linear and the other supercoiled, only a linear dimer is produced (Abremski et al., 1983). The presence of a similar recombination system on F has not at present been demonstrated, but it is likely that for such unit-copy plasmids maintenance of monomeric conformation will be vital to the process of efficient plasmid partitioning, and therefore would suggest the existence of a similar system encoded by F.

1.3 Control of cell division in E.coli K-12

1.3.1 Introduction

Perturbations in E.coli DNA synthesis have been demonstrated to elicit abrupt physiological changes, collectively known as the 'SOS response'. This response includes the induction of systems associated with DNA repair and mutagenesis, inhibition of cell division, a reduction in respiration, the induction of resident prophages, restriction alleviation, and stable DNA replication (Table 1.3), (Little & Mount, 1982).

The molecular basis of the SOS response and its regulation are now at least partly understood. The presently accepted model of the 'SOS response' includes two key regulatory components, the recA and lexA gene products (Fig.1.23) (Walker, 1984). In an uninduced cell the lexA gene product functions as a repressor of a number of unlinked genes, some of which are listed in Table 1.3. These genes comprise the SOS regulon and include the recA and lexA genes themselves, indicating that the LexA polypeptide acts as an autogenous repressor. Some loci, such as recA, have a single site at which the LexA repressor binds, while others, such as lexA itself, have two such sites. In addition, many of the SOS genes, including the recA and lexA genes, are expressed at low levels in the uninduced state. Perturbations of DNA replication generate an inducing signal that leads to the proteolytic digestion of the LexA protein, as well as a small number of other proteins, such as the phage lambda repressor. Proteolysis is catalysed by a reversible

Induced physiological response
or gene function

Induced genes

E.coli K-12:

Prophage induction	<u>umuDC</u> , <u>uvrA</u> , B and C
U.V.mutagenesis	<u>umuDC</u> , <u>recA</u>
Filamentation (inhibition of cell division)	<u>sulA</u> (<u>sfiA</u>)
<u>uvrA</u> ⁺ <u>B</u> ⁺ <u>C</u> ⁺ -dependent repair	<u>uvrA</u> , B and C
Long patch repair	<u>uvrA</u> , B and C
RecF-dependent recombination	<u>recN</u>
Hyper-recombination in <u>uvrD</u> strains	?
Inhibition of DNA degradation by exonuclease V	<u>recA</u>
Induced radioresistance	<u>recA</u>
Repair of double-strand breaks	<u>recA</u>
Induction of RecA protein (roles in homologous recombination & specific protease involved in SOS regulation)	<u>recA</u>
Induction of LexA protein (repressor, role in SOS regulation)	<u>lexA</u>

Table 1.3

Some of the functions expressed by and genes contained within the SOS regulon. (Reproduced from Walker, 1984).

Table 1.3 Con't.

Induced physiological response or
gene function

Induced genes

Induction of HimA protein (part of integration host factor, role in site-specific recombination)	<u>himA</u>
Induction of UvrD protein (helicase II, roles in excision repair & methyl-directed mismatch repair)	<u>uvrD</u>
Induction of single-strand DNA-binding protein	<u>ssb</u>
Induction of <u>ruv</u> locus (unknown role in U.V.resistance)	<u>ruv</u>

Naturally occurring plasmids:

Colicin production	Colicin E1 (ColE1)
Cloacin production	Cloacin DF13 (CloDF13)

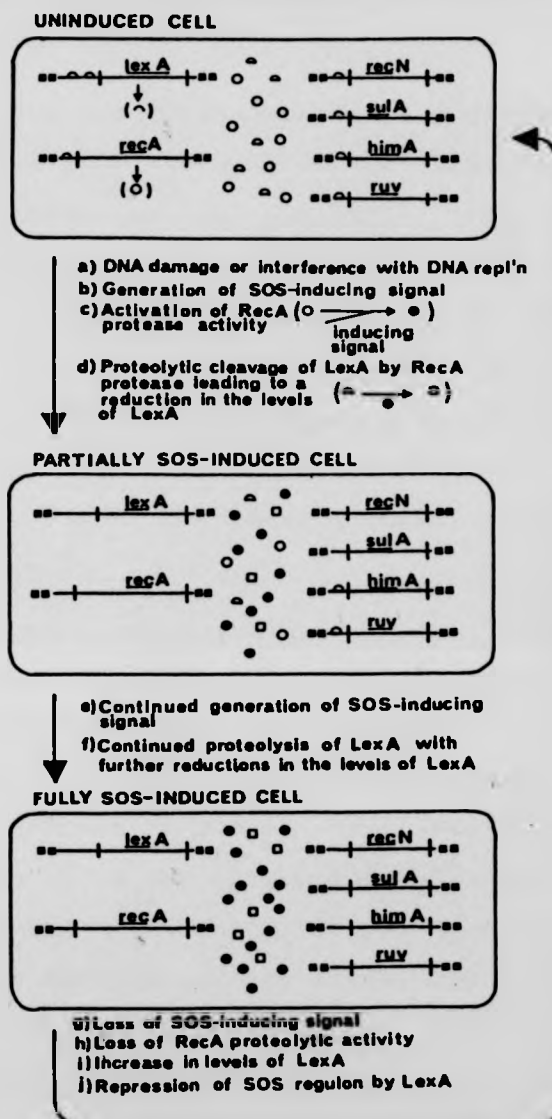


Figure 1.23

Schematic representation outlining stages involved in the induction of the SOS regulon. The extent of derepression of the SOS system, depends on the strength of the inducing signal (Adapted from Walker, 1984).

specific protease activity, associated with the RecA protein. As the RecA proteolytic activity reduces LexA-mediated repression, various SOS genes, including the recA gene, are increasingly expressed. The degree of this expression is related to the strength of the inducing signal. Recovery, associated with the repair of damaged DNA, eliminates the inducing signal returning RecA molecules to a proteolytically inactive state. This leads to increased levels of the LexA repressor and a return to the uninduced state.

1.3.2 Pathways of division inhibition in E.coli K-12

Termination of a round of chromosome replication has been suggested as being an essential pre-requisite for the cell division process to occur. Additionally, it has been shown that a short period of protein synthesis, following termination of DNA replication, is also necessary, indicating that termination may signal the transcription and translation of specific sequences which play a role in the initiation of cell division (Jones & Donachie, 1973). It has recently been proposed that the ftsA gene product may function as such a termination protein (Tormo et al., 1980 & 1985). The ftsA coding sequence lies within a region of the E.coli genome located at two minutes, which contains a number of cell division genes (Fig.1.24), (Sullivan & Donachie, 1984). In particular, it has been shown that the ftsA gene lies immediately adjacent to the essential cell division gene ftsZ (sfiB, sulB), whose gene product is thought to be necessary for the initiation of septum formation (Lutkenhaus, 1983). Furthermore, promoter

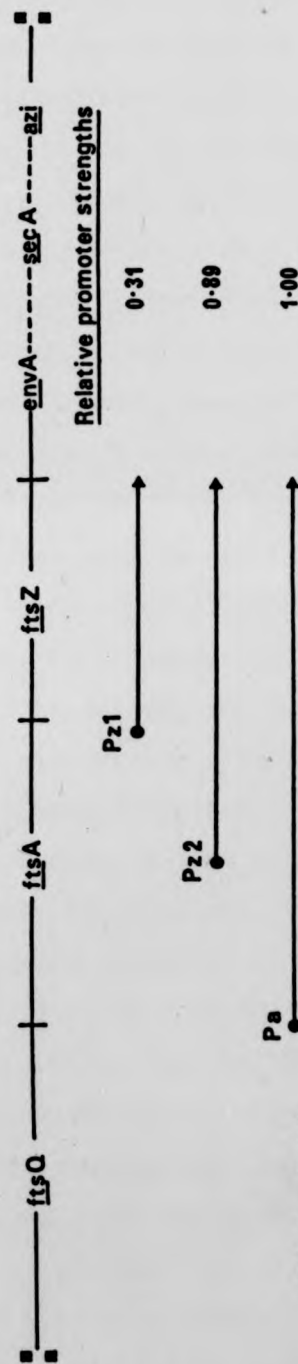


Figure 1.24

Diagrammatic representation of promoter activity within the *ftsA* and *ftsZ* coding region. This region is located within a cluster of cell division genes, which map at two minutes on the *E. coli* K-12 genome. Direction of transcription is indicated by the arrows, and the strength of transcription initiated from promoters transcribing into the *ftsZ* region (*Pz1* and *Pz2*), are shown as a relative value of the transcriptional strength of the *ftsA* promoter (*Pa*), which also co-transcribes the *ftsZ* region.

(Adapted from Sullivan & Donachie, 1984).

activity originating within the ftsA coding sequence has been shown to lead to higher levels of transcription of the ftsZ gene, suggesting a direct link between the termination of DNA replication and cell division (Fig. 1.24), (Jones & Holland, 1984; Sullivan & Donachie, 1984). However, this does not rule out the possibility that other cell division genes may also be involved in linking termination of DNA replication with cell division. Indeed, newly identified cell division mutants of E.coli have been located near to the terminus of chromosome replication (Dwek et al., 1984; Bejar & Bonche, 1985). Further studies will, however, be required in order to determine their functional relationship to the cell division process.

The SOS system defines two important functions that are relevant to the bacterial cell cycle, a) restitution of genome integrity as a consequence of DNA damage repair processes, and b) inhibition of cell division until DNA repair or replication can be completed. Recognition that division inhibition constitutes one of the various functions of the SOS system, has lead to the hypothesis that subsequent to DNA damage a specific inhibitor(s), of cell division is synthesized (Radman, 1975). The mutations tif and tsl, represent defects expressed at 42°C in the recA and lexA gene products respectively, proteins which control the induction of SOS functions. Both tif and tsl cells filament at 42°C. Extragenic suppressors of these mutations, designated sfiA and sfiB, have been isolated and are thought to define genes specifically involved in the SOS pathway of division inhibition

(Huisman & D'Ari, 1983). Recently, it has been demonstrated that the sfiA gene undergoes derepression following SOS inducing treatments. In particular, it has been shown that the initial rate of sfiA expression is proportional to the U.V.-light dosage and was identical in either a wild-type uvr or uvrA background (Quillardet & Hofnung, 1984). The nucleotide excision repair pathway of E.coli, incorporating the uvrA, B and C genes, represents a primary prokaryotic cell defence strategy against U.V.-light mediated lesions in DNA (Walker, 1984). Therefore, these findings indicate that the sfiA gene may encode the postulated division inhibitor associated with DNA repair, and that the initial rate of sfiA expression appears to be determined by the number of U.V.-light generated DNA lesions, before the occurrence of any repair associated reduction in the number of these lesions. Studies involving the expression of a sfiA-lac operon fusion, have demonstrated that neither sfiA nor sfiB regulates the synthesis of sfiA. Instead, two mutations, infA and infB, have been identified and may have some role in the regulation of sfiA expression (Huisman & D'Ari, 1983).

The presence of a second pathway of division inhibition, similarly dependent on a functional recA gene product but independent of both sfiA and sfiB, has recently been demonstrated (Burton & Holland, 1983). This second pathway, in contrast to the sfi-dependent pathway, is resistant to rifamycin and only functions in the presence of a DNA replication fork (Fig.1.25). It was therefore proposed that E.coli possesses two pathways of

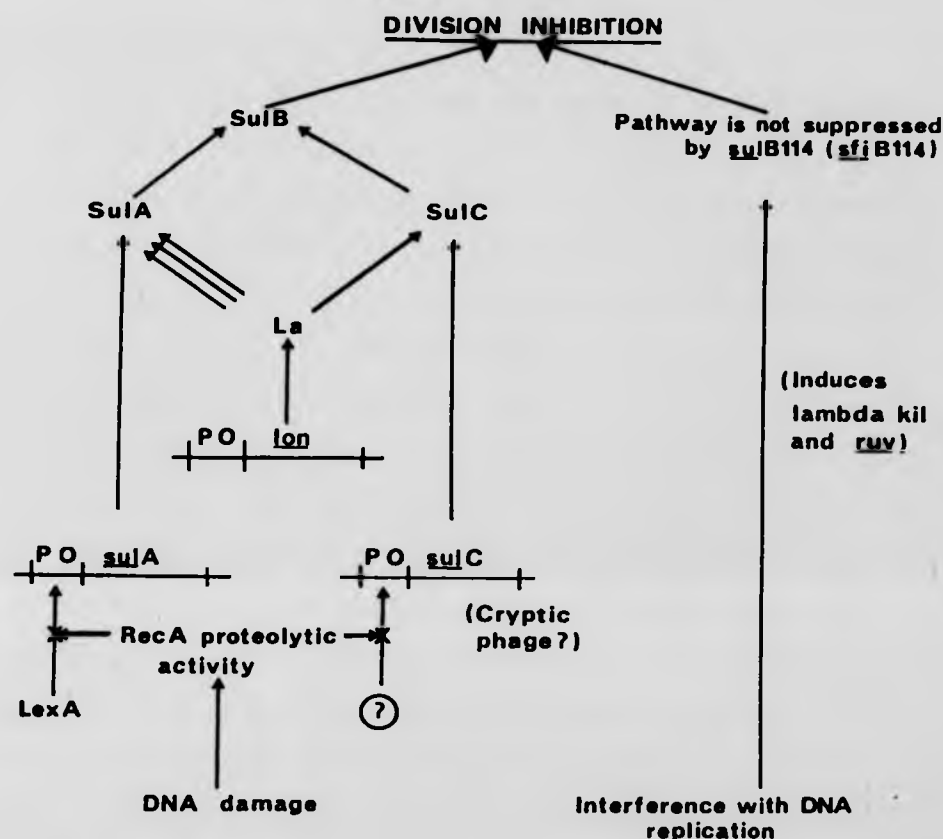


Figure 1.25

Schematic representation of the sfi (sul)-dependent, and independent pathways of division inhibition occurring in E.coli K-12 strain AB1157. Other strains of E.coli K-12 may be expected to differ with respect to expression of the division inhibitor sulC, which may be encoded by a cryptic prophage. The relative inhibitory activity of protease La on the division inhibitors SulA and SulC, is indicated by the number of arrows. SulB represents the product of the essential cell division gene ftsZ, on which the gene products of either SulA or SulC act to inhibit cell division.

division inhibition. The sfi-dependent pathway, representing division inhibition mediated as a consequence of an SOS induced response and able to operate over the entire E.coli cell cycle, and the sfi-independent pathway, functioning to inhibit cell division as part of the normal coupling between chromosome replication and cell division and operating over the C-period only. Little is known at present concerning the specific nature of the sfi-independent pathway, other than evidence pointing to its existence.

In contrast, some molecular details have emerged over the past few years concerning the more experimentally amenable sfi-dependent pathway. Analysis of mutations occurring in the lon (capR) gene of E.coli, clearly indicate an involvement for this gene product in a wide variety of cellular processes. In particular, lon strains form long nonseptate filamentous cells following treatments that damage DNA (U.V.-light irradiation, nitrofurantoin, etc.), or inhibit DNA replication (nalidixic acid, thymine starvation, etc.). Even shifting from minimal to rich medium induces filamentation in lon mutants (Gayda et al., 1976). In addition, lon cells show decreased degradation of missense and nonsense proteins, as well as certain wild-type proteins (Gottesman & Zipser, 1978; Shineberg & Zipser, 1973; Gottesman et al., 1981a), they are defective in F maintenance and P1 lysogeny (Falkinham, 1979; Takano, 1971), lambda lysogeny (Walker et al., 1973), and overproduce capsular polysaccharide (Markovitz, 1977).

The lon gene product of E.coli K-12 has been identified as the 94kDa polypeptide, the monomer component of the homotetrameric protein, protease La, a serine protease localized to the cytoplasm (Schoemaker & Markovitz, 1981; Chung & Goldberg, 1981; Swamy & Goldberg, 1982). Protease La possesses, as determined by in vitro studies, multiple activities that may be related to the lon phenotype. These comprise a proteolytic activity requiring ATP hydrolysis, a nonspecific nucleic acid binding activity, and a DNA-stimulated ATPase activity (Chung & Goldberg, 1982). Studies of the capR9 allele of lon indicate that the defective polypeptide has lost both protease and ATPase activities, but retains the nonspecific nucleic acid binding activity (Charette et al., 1984). In vitro mixing of purified CapR9 and wild-type CapR polypeptide monomers in a 4:1 ratio, indicates that the CapR9 protein is able to associate into a tetrameric form with the wild-type polypeptides, inhibiting both the protease and proteolytic ATPase activities by about 50% (Fig.1.26). In contrast, the presence of only one CapR9 monomer, as presumed from the ratio mixture of wild-type to defective monomers, is sufficient to inhibit the DNA-stimulated ATPase activity. Furthermore, when protease and ATPase activities are monitored in the absence and increasing presence of DNA, proteolysis decreases as ATPase activity increases, indicating the existence of two ATPase activities, one protein stimulated, the other DNA-stimulated which function simultaneously (Fig.1.26). Nothing is at present known as to the role of protease La with respect to the

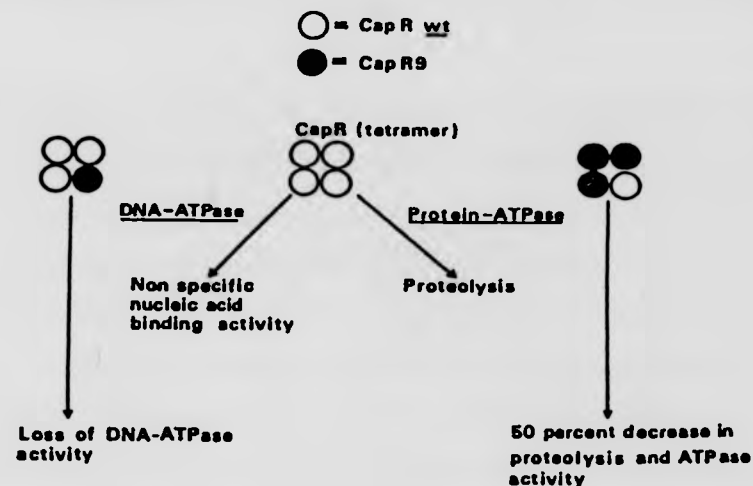


Figure 1.26

Schematic representation of in vitro observations of the ATPase activities of the Lon (CapR), protein tetramer, protease La. Purified monomers of wild-type (capR), and mutant allele (capR9), gene products were mixed, and assayed for either DNA, or protein-stimulated ATPase activities. ATP hydrolysis, stimulated by nonspecific nucleic acid binding, occurs maximally in the presence of supercoiled (form I) DNA. In a 3:1 ratio of CapR to CapR9 monomers, the DNA-stimulated ATPase activity is inhibited. A 1:3 ratio of CapR to CapR9 monomers, reduces the protein-stimulated ATPase activity by about 50 percent. DNA and protein competitively bind to the capR gene product. These in vitro observations may explain differences in the phenotype of capR strains bearing either an F, or pSC101 plasmid derivative encoding the capR9 mutant allele. The F' bearing strain exhibits only a mucoid phenotype, while the pSC101 recombinant strain exhibits both a mucoid and filamentous phenotype, presumably reflecting the differences in copy number of the plasmids and therefore gene dosage of the capR9 allele.

DNA-stimulated ATPase activity, other than that it neither involves phosphorylation, nor adenylation of protein, nor does it lead to the relaxation of supercoiled DNA, in whose presence a maximum rate of ATP hydrolysis occurs, nor does it function as a nuclease. It has been suggested that protease La may use the energy of ATP hydrolysis to migrate along DNA searching for specific proteolytic targets, other than the RecA protein or the A and B subunits of DNA gyrase which appear to be unaffected (Charette et al., 1984).

Protease La, therefore, may function in one of two distinct ways so as to mediate its action on the various cellular components associated with the lon phenotype. The first and most direct mode of regulation may involve the possibility of protease La acting as a classical repressor of transcription. Studies on the regulation of capsular polysaccharide synthesis has provided some evidence to substantiate this mode of action. In particular it has been shown that for the galactose operon, increased levels of mRNA can be detected in capR mutants, suggesting the existence of a second operator within this operon responding to capR control (Markovitz, 1977). A second and indirect mode of regulation may involve proteolytic activation or inactivation of substrates. For instance, the functional half-life of the lambda N gene product increases several-fold in capR cells (Gottesman et al., 1981a), while the precursor of the outer membrane protease OmpT, is considerably more stable in capR strains (Gordon et al., 1984). Levels of OmpT, a trypsin-like protease, have been correlated with the

synthesis of capsular polysaccharide (Gayda et al., 1979). In addition, it has recently been shown that at least five genes (cps A,B,C,D & E), are responsible for capsular polysaccharide synthesis, all of which are negatively regulated by the lon gene product (Trisler & Gottesman, 1984). Three additional genes have been identified which regulate cps gene expression, these are the rcaA and B genes that function as positive regulators, and the rcaC gene that functions as a negative regulator (Gottesman et al., 1985). Evidence has suggested that it may be the rcaA gene product which is regulated by protease La. Finally, the postulated division inhibitor SfiA (SulA), is known to have an increased stability in lon strains (Mizusawa & Gottesman, 1983). This observation may go some way in explaining the associated filamentation of lon cells, but as with the other polypeptides mentioned above, it remains to be determined what the exact nature of the interaction of protease La is with the SfiA (SulA) protein.

Recently, it has been demonstrated that protease La is a component of the heat shock (HTP) regulon, which consists of at least seventeen genes primarily subject to their own individual controls but additionally having a co-ordinated response to elevated temperatures. This response is dependent on the product of the htpR regulatory gene situated at 76 minutes on the E.coli chromosome (Phillips et al., 1984). The identification of protease La as a member of the HTP regulon, therefore establishes a possible link between the HTP and SOS stress response systems of E.coli.

Extragenic suppressors of lon, designated sul, have been isolated as U.V.-light resistant, or methyl methane-sulphonate-resistant (MMS) derivatives. These lon derivatives are not altered with respect to their mucoid and protein degradation properties. Analysis of sul mutations has revealed that they are located on the E.coli genome at two loci, sulA at 22 minutes and sulB at 2 minutes and are allelic with sfiA and sfiB respectively, as determined by indistinguishable phenotypes and chromosome location (Gottesman et al., 1981b). The sulA⁺ gene product is an 18kDa polypeptide not only induced by treatments that damage DNA, such as U.V.-light irradiation, but also by agents that inhibit DNA replication such as nalidixic acid. The Sula protein, whose coding region does not contain a signal sequence, has been shown to be associated with outer membrane fractions of E.coli (Schoemaker et al., 1984). The Sula protein present in these fractions is not proteolytically degraded by protease La, even though the conditions employed led to the proteolysis of pro-OmpT. It was therefore concluded that the Sula protein may not be a direct substrate for protease La, and that protease La negatively regulates either the concentration or activity of Sula through an undefined proteolytic action.

In addition to the sulA gene product at least one other protein has been suggested as a candidate cell division inhibitor. This 11kDa protein, designated 'polypeptide a', is also apparently under the control of protease La, but independent of the SOS response. Its synthesis was detected in a lon (capR82) recA strain of

E.coli K-12, when cell division was inhibited following a nutritional shift from minimal to rich medium. It was therefore concluded that 'polypeptide a' could either be a protein involved in cell division inhibition, or a precursor of a protein required for cell division (Schoemaker *et al.*, 1982).

The isolation of sulB mutations is relatively rare in comparison to sulA, no insertion mutations have been isolated and sulB mutations are dominant over wild-type. In a wild-type lon background, sulB mutations still cause cell division defects. These cells are sensitive to low ionic strengths and high growth temperatures. They exhibit aberrant cell division, when grown in rich medium containing 1/10th the normal concentration of sodium chloride at 42°C (Lutkenhaus, 1983). These observations suggest that sulB may encode an essential cell division gene product, possibly coding for an enzymatic activity that may modify the cell wall allowing septum formation (Gottesman *et al.*, 1981b; Lutkenhaus, 1983). Furthermore, it has been proposed that the sulB gene product is the target of the Sula division inhibitor. Indeed, recent studies involving a lacZ-ftsZ (sulB) gene fusion have shown that the lacZ-ftsZ fusion polypeptide, designated ZZ, when induced does not possess ftsZ activity, but instead behaves as an analog of the cell division inhibitor sulA (Ward & Lutkenhaus, 1984). Division inhibition on induction of the ZZ polypeptide was found to be circumvented by an increase in the gene dosage of either ftsZ, or the sulB allele of ftsZ known to code for an altered but functional FtsZ protein resistant to Sula

activity. These findings suggest that the ftsZ gene product functions as a multimer, and the presence of both FtsZ and the ZZ polypeptides leads to the formation of a nonfunctional mixed multimer. However, an increase in the concentration of FtsZ or SulB with respect to the ZZ polypeptide, results in the formation of a significant concentration of either a normal wild-type multimeric complex of FtsZ, or respectively, the sulB gene product that is resistant to complex formation with ZZ. It remains, however, to be seen whether further studies on this FtsZ-hybrid protein may in due course explain the mechanism by which Sula inhibits cell division.

sulA (sfiA), does not appear to be the only gene product capable of interacting with FtsZ. It has recently been demonstrated that certain E.coli strains encode a system of division inhibition associated with the sfi-dependent pathway, but expressing a unique division inhibitor termed sfiC (sulC), (Fig.1.25), (D'Ari & Huisman, 1983). The sfiC gene product exhibits a number of properties similar to those of sfiA, both being associated with the induction of the SOS system as a consequence of expression of the recA (tif) mutation. Both are suppressed by sfiB and amplified in strains lacking protease La. However, sfiA and sfiC differ with respect to the extent of amplification in lon strains, their respective E.coli map positions (sfiC is located at 28 minutes), and their regulation (sfiC does not appear to be under the direct negative control of the LexA repressor). It has been concluded that sfiC may represent an inducible cryptic prophage gene, the repressor of which is

susceptible to RecA proteolytic activity.

Finally, in addition to sfiA and sfiC, a separate defect designated tsM, appears to specify a function which directly or indirectly leads to division inhibition via a postulated interaction with FtsZ (Drapeau *et al.*, 1984). tsM containing strains exhibit phenotypic characteristics, albeit expressed to a much lower extent, similar to those of lon strains. Cells carrying the tsM defect form long nonseptate filament cells at 42°C, and will also filament extensively following a nutritional shift-up. They have a reduced colony forming ability on rich but not on minimal medium following U.V.-light irradiation, and are deficient in lysogenization by bacteriophage lambda, tsM containing strains also produce mucoid colonies (Belhumeur & Drapeau, 1984). Filamentation, brought about by the tsM defect, is not suppressed by the sfiA11 allele. In addition, it appears that two levels of regulation of the tsM defect may be present, one dependent on wild-type lexA and expressed as a result of the induction of the SOS system, the other lexA-independent and possibly induced by agents associated with a nutritional shift. Furthermore, the tsM defect has been shown to be located at two minutes on the E.coli chromosome, but appears to be distinct from the cluster of division genes present within this region (Drapeau *et al.*, 1984).

1.4. Recombination pathways associated with DNA repair and plasmid recombination.

1.4.1 Introduction

The SOS response of E.coli maintains the integrity of the genetic information of the cell against agents or conditions that would either disrupt the structure of the host genome(s), or obstruct its replication. This is primarily achieved by the inducible expression of two important functions, namely, inhibition of the cell division process and enhancement of DNA repair (Little & Mount, 1982). Expression of the latter greatly affects the mutagenic consequences of different DNA-damaging treatments, and is correlated with an enhanced mutagenesis (Witkin, 1976).

For E.coli strains which are defective in excision repair (i.e., uvr), the foremost dark repair system operating is that of postreplication repair. The current hypothesis accounting for this repair process proposes that daughter strand gaps are formed in newly synthesized DNA, as a consequence of the inability of the replication complex to correctly base pair with U.V.-light induced lesions (i.e., pyrimidine dimers), which have not been removed by the excision repair process and are contained within the DNA template strand (Fig.1.27). Since the excision repair process is unable to correct U.V.-lesions at daughter strand gaps, the gaps are subsequently filled by a recombinational process. This process requires functional RecA and the complementary DNA strand located nearby in the sister DNA duplex, that was formed by the

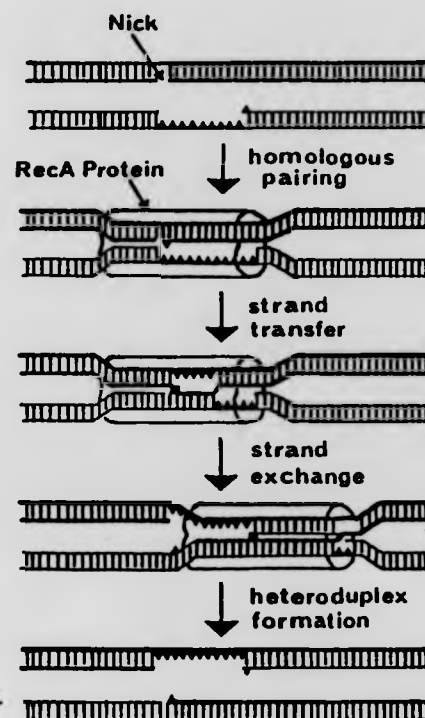


Figure 1.27

Schematic representation of RecA-mediated joint molecule formation and strand exchanges between gapped and nicked duplex DNA (postreplication repair). The formation of heteroduplex DNA molecules involves homologous pairing at the gapped site and is followed by reciprocal strand exchange. It is assumed that following heteroduplex formation, the resultant structure is repaired by DNA polymerase and ligase functions.

(Reproduced from West *et al.*, 1982).

same replication fork. The used complementary strand from the 'sister duplex' is replaced by DNA synthesis, and the remaining unrepaired dimer will now be accessible to the excision repair process (Fig.1.27), (Rupp & Howard-Flanders, 1968; West et al., 1982).

The RecA protein, apart from its proteolytic role in regulating the SOS response, is also essential for general recombination occurring in E.coli. A mutation in recA gives a several-fold logarithmic reduction in recombination. RecA is also able to function, albeit in vitro, as a DNA-dependent ATPase that promotes homologous pairing of DNA molecules. The ATPase activity resides in a tetrameric form of RecA protein monomers, each of which are about 37kDa (Radding, 1981). It has been proposed that there are two important independent pathways of recombination, associated with postreplication repair in wild-type strains of E.coli, both requiring wild-type recA. The first of these is the RecBC pathway, which in addition to being dependent on the recB and recC genes that together encode exonuclease V, is also dependent on the uvrD and lexA gene products (Wang & Smith, 1983). However, the RecBC pathway appears to play only a minor role in the repair of daughter strand gaps. Experimental evidence suggests that one possible mechanism of recombination associated with the RecBC-dependent pathway of post-replication repair, may involve a process in which double-stranded DNA exchanges occur between two DNA molecules. This repair process has been termed 'sister duplex recombination', to distinguish it from the hypothesized 'daughter-strand gap filling' repair process

(Wang & Smith, 1983). This latter process of 'daughter-strand gap filling' appears to rely largely on a minor homologous recombinational pathway which requires the recF, recN, recQ1, rec-259, recJ and lexA genes (Wang & Smith, 1983; Nakayama *et al.*, 1984). This RecF-dependent pathway in recBC mutants is enhanced by an additional mutation in the sbcB gene, the structural gene for the 53.7kDa polypeptide exonuclease I (Prasher *et al.*, 1983). This enzyme mediates degradation of single-stranded DNA, an intermediate suggested to be utilized by the RecF-dependent pathway during recombination.

The RecBC and RecF pathways not only function to mediate postreplication repair, but also appear to play direct roles in the generation of the SOS signal, leading to the induction of recA proteolytic activity and synthesis. It has been demonstrated that the synthesis of recA mRNA is greatly stimulated by agents that lead either to DNA-damage, or the inhibition of DNA replication. Mutations in either the recB or recC loci prohibit the induction of recA mRNA synthesis following nalidixic acid treatment (Fig.1.28), (McPartland *et al.*, 1980). On the other hand, mutations in recF markedly reduce the U.V.-light inducibility of recA mRNA production (Fig.1.28). Since significant levels of recA mRNA can also be detected in recF recBC double mutants, following U.V.-light irradiation, it would appear that an alternative route(s) also functions to mediate U.V.-light induced recA mRNA synthesis. Finally, in addition to the RecBC and RecF pathways of homologous recombination, mutational analysis has demonstrated the existence of a

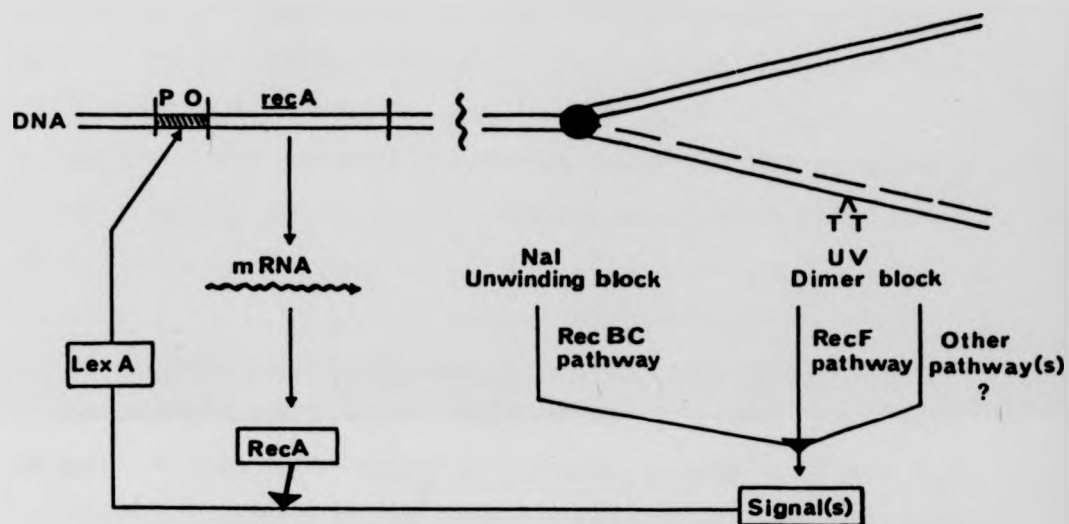


Figure 1.28

Postulated model of the early events occurring during induction of the SOS regulon. The RecBC, or the RecF pathways (and possible additional pathways), respectively mediate generation of the *recA* inducing signal, following either, inhibition of chromosome replication by nalidixic acid (Nal, unwinding block), or U.V.-light production of pyrimidine dimers (dimer block).

(Reproduced from McPartland *et al.*, 1980).

third pathway of recombination, presumed to function less effectively than the RecF pathway, which is present in E.coli strains lysogenic for the Rac prophage. The Rac genome encodes recE, the determinant for exonuclease VIII. sbcA mutations (suppressors of either recB or recC mutations), are assumed to occur within the Rac prophage, leading to the constitutive expression of RecE (Willis et al., 1983).

1.4.2 Plasmid recombination

Plasmid recombination is primarily assumed to involve an interaction between two covalently closed circular DNA molecules. Recombination between plasmid molecules occurs as a consequence of either a recA-dependent process, using several components associated with general recombination, or a recA-independent site-specific recombination process (Bedbrook & Ausubel, 1976). This latter process mainly involves recombination systems that lead to the resolution of co-integrate structures, normally catalysed by recombinase enzymes at recombination specific sites encoded within either transposable elements or plasmids (Calos & Miller, 1980; Dodd & Bennett, 1983; Summers & Sherratt, 1984; Hoess & Abremski, 1984; Hakkaart et al., 1984).

Since the isolation of intact plasmid DNA subsequent to recombination enables analysis of plasmid configuration, inter- and intraplasmidic recombination has been studied with respect to the RecBC, RecF and RecE pathways. Formation of plasmid multimers in E.coli occurs

readily at a high frequency in wild-type rec strains, to a lesser extent in recBC strains, and is dramatically reduced in recA and recF strains (James *et al.*, 1982; Cohen & Laban, 1983). In general, plasmid multimers formed by homologous recombination have been shown to be covalently closed circular DNA molecules composed of tandemly repeated plasmid monomers (James *et al.*, 1982). They appear to be derived, following the maturation of a recombination intermediate formed between two homologous plasmid molecules. The recombination intermediate is observable by electron microscopy, and has been termed an 'Holliday structure' (Fig.1.29), (Potter & Dressler, 1977). Two alternative pathways have been proposed for the resolution of 'Holliday intermediates' giving rise to the formation of either two plasmid monomers, or a single plasmid dimer. These alternative pathways of maturation depend on which of the four DNA strands are cleaved at the 'Holliday junction', linking either two plasmid monomers, or resolving a plasmid dimer. These pathways represent either inter- or intramolecular recombination events respectively (Fig.1.30), (Potter & Dressler, 1977).

For plasmid recombination, the products of the recA and recF genes appear to be a main requirement (Cohen & Laban, 1983). However, recB strains which carry the Rac prophage and an abcA mutation are also known to exhibit recF-independent plasmid recombination, presumably as a consequence of RecE induction (Fishel *et al.*, 1981). These and other studies have also concluded that there is a fundamental difference between inter- and intraplasmidic recombination, as demonstrated by the different effects of

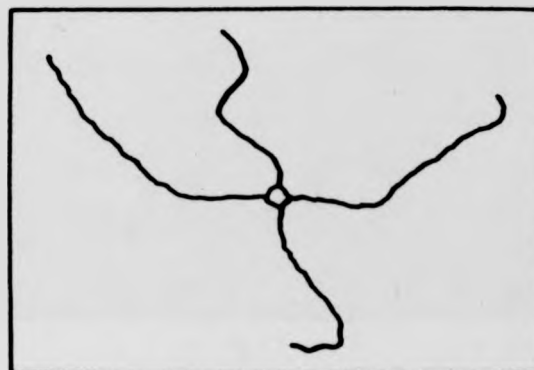


Figure 1.29

Line traced diagram of an electron micrograph of a 'Holliday structure'. The structure is observed following the linearization of plasmid DNA recombination intermediates with a restriction endonuclease.

(Reproduced from Potter & Dressler, 1977).

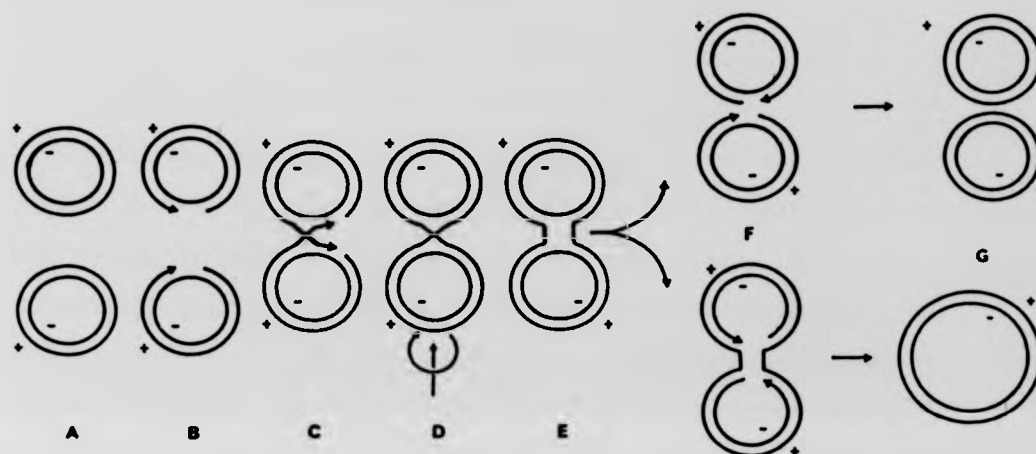


Figure 1.30

Schematic representation of two identical plasmid DNA molecules, undergoing recombination via an Holliday intermediate. Maturation of the Holliday intermediate (E), may occur via one of two pathways. The upper pathway leads to the formation of plasmid monomers, while the lower pathway leads to the generation of a plasmid dimer. The final product is dependent on the choice of DNA strands for cleavage and ligation (F).

(Adapted from Potter & Dressler, 1977).

recBC recF double mutations (Cohen & Laban, 1983), and by the roles of the recA and recBC gene products on recombination involving the recE pathway (Fishel *et al.*, 1981; Laban & Cohen, 1981). Plasmid recombination, therefore, involves one of two recombinational interactions defining either an inter- or intraplasmidic recombination event. General recombination may be catalysed by either the RecF or RecE pathways of recombination. The RecE pathway of intraplasmidic recombination appears to be the only pathway independent of RecA (Fishel *et al.*, 1981; Laban & Cohen, 1981).

To summarize, it seems that the RecBC, RecF and RecE pathways define alternative pathways of recombination in E.coli K-12, and that the functional nature of the pathways are determined by the structure of the recombination substrates. However, the RecF pathway appears to be the major pathway associated with plasmid recombination.

1.5 Continuous culture as applied to the study of plasmid stability

1.5.1 General principles of continuous culture

Any particular nutritional component which a bacterial strain requires for growth may be referred to as a growth nutrient. The absence of a single growth nutrient will result in no growth. There also exists a range over which a sufficiently low concentration of an added growth nutrient will determine the growth-rate. The maintenance

of such a 'limiting nutrient' at a concentration allowing control of the growth-rate of a bacterium, while all other growth factors are in excess, is the basic principle of continuous culture.

In 1950, details were independently published of two similar devices capable of maintaining a 'limiting nutrient' at a specific concentration. Both these devices, the 'chemostat' (Novick & Szilard, 1950a), and the 'bactogen' (Monod, 1950), operate by controlling the flow-rate of a simple sterile medium containing a 'limiting nutrient', through a culture vessel in which a constant culture volume is maintained by simply allowing overflow to occur. A constant overflow ensures that exhausted medium and excess bacteria leave the culture vessel at the same rate at which fresh medium flows in (Fig.1.31). In addition to a controlled flow-rate, it is essential that the culture vessel should be thoroughly stirred, so that fresh medium flowing in is quickly dispersed within the culture. For such an apparatus the average time that a particle resides in the culture vessel, termed the 'mean residence time', will be determined by the ratio of the flow-rate and vessel volume (Herbert, 1958), which is termed the 'dilution rate', D , defined as:

$$D = \frac{f}{v} = \frac{\text{flow-rate}}{\text{volume}} \quad (\text{dimensions: hours}^{-1})$$

Therefore, if the rate of flow of medium is held constant then such an apparatus should automatically

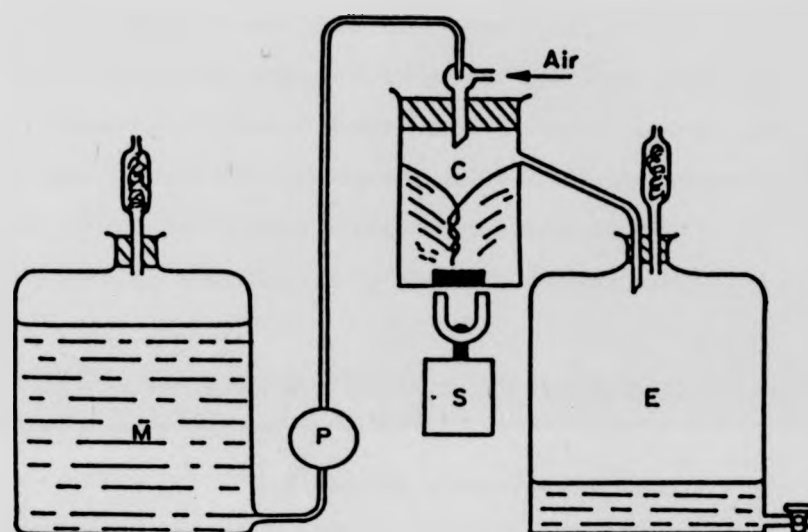


Figure 1.31

Diagrammatic representation of the essential features of a chemostat. Sterile medium (M), held within a reservoir, is added to the culture vessel (C), at a constant rate determined by a peristaltic pump (P). The culture is agitated by a magnetic stirrer (S), and aerated by pumping sterile air through into the culture vessel. Constant culture volume is maintained by a constant level overflow into an effluent reservoir (E).

(Reproduced from Tempest, 1970).

establish a 'steady state', in which the concentration of bacteria and substrate remains constant as long as the dilution rate is unaltered. Today the term 'chemostat' has been adopted to describe such continuous culture devices. These devices may in addition to the above simple essential features, also include other apparatus to control such culture parameters as temperature, pH, dissolved O₂ concentration etc.

1.5.2 Growth kinetics of bacteria in batch and continuous culture

The attainment of a 'steady state' within a chemostat culture represents a situation where the specific growth-rate of a bacterial population, that is the rate of increase of organism concentration with time, expressed in any arbitrary value normally denoted by the symbol μ , equals the dilution rate. However, in practice it is not always possible to obtain an ideal 'steady state' since, a) not all cells will be exposed to an identical culture environment as complete instantaneous mixing of inflowing nutrients does not occur, and b) bacterial cultures are subject to mutational change leading to selection as a consequence of the changes affecting specific growth-rate parameters (Kubitschek, 1972). In spite of such discrepancies between the theory and practice of continuous culture, the kinetics of steady-state bacterial growth can be accurately described using mathematical formulae.

All continuous cultures commence existence with a batch culture. Batch culture is characterized by a

sequence of changes that have been referred to as the growth cycle. Such growth cycles normally commence with a lag phase, during which time cells adapt to a new nutritional environment, their mass increases and eventually they divide. This is followed by a period of growth where cell numbers increase exponentially, termed the exponential or log phase. Finally, the culture enters stationary phase, where growth and division cease as a consequence of the depletion of a nutrient(s), or the accumulated presence of an inhibitory factor(s). Therefore, during batch culture cell properties would be expected to vary considerably. However, during the exponential growth phase average cell properties remain constant, since culture biomass doubles at a relatively constant rate (Tempest, 1970). This can be described mathematically as:

$$\frac{1}{x} \frac{dx}{dt} = \frac{d(\ln 2x)}{dt} = \mu = \frac{\ln 2}{t_d} \quad (1)$$

where: x = the concentration of organisms.

t = time

μ = the specific growth-rate, which is the rate of increase per unit of organism concentration.

t_d = the doubling time (i.e., the time required for the concentration of organisms to double).

Generally both μ and t_d are constants, but they can be markedly influenced, particularly by the

concentration of an essential limiting nutrient. Indeed, it has been demonstrated that there is a simple relationship between the specific growth-rate of a bacterium and the concentration of an essential nutrient (Monod, 1942). In essence, μ is proportional to the limiting nutrient concentration, but reaches a saturation value at high concentrations of this nutrient. The dependence of μ on limiting nutrient concentration was shown to be analogous to the enzyme kinetic function of Michaelis-Menten (Monod, 1942 & 1950), and states that:

$$\mu = \mu_{\max} \left(\frac{S}{K_s + S} \right) \quad (2)$$

where: S = the substrate or limiting nutrient concentration.

μ_{\max} = the growth-rate constant (i.e., the maximum value of μ at saturation levels of substrate).

K_s = a saturation constant equal to the concentration of limiting substrate or nutrient, allowing growth at one half its maximal rate (i.e., $\mu = \frac{1}{2}\mu_{\max}$).

In batch culture, however, substrates are consumed as cells grow through the exponential phase, therefore, S decreases and so eventually will μ , until there is insufficient concentration of the limiting substrate to support growth of the cells. In contrast, the chemostat, due to the constant addition of fresh medium, maintains a specific concentration of the limiting substrate, which therefore fixes the specific growth-rate of the cells at some point less than μ_{\max} . Therefore, the relationship between dilution rate and the specific growth-rate of an organism is an important parameter of the chemostat.

Consider then the culture vessel of a chemostat, within this vessel organisms will be growing and at the same time being washed out through the overflow. The net change in concentration of cells (x), with time, may be represented as:

Increase = Growth - output

$$\frac{dx}{dt} = \mu x - Dx$$

$$\frac{dx}{dt} = \mu(x-D) \quad (3)$$

Therefore, if $\mu > D$ then dx/dt will be positive, and the concentration of organisms in the culture vessel will increase with time. However, if $\mu < D$ then dx/dt will be negative, and the concentration of cells in the

culture vessel will decrease (i.e., the culture will experience 'wash-out' from the culture vessel). When $\mu=D$, then dx/dt will be zero, and the cell density will remain constant with time (i.e., the culture will have attained a 'steady-state'), (Tempest, 1970).

Therefore:

$$D = \mu = \mu_{\max} \left(\frac{S}{K_s + S} \right) = \frac{\ln 2}{t_d}$$

The 'generation time' or 'doubling time' (t_d) of the culture is therefore equal to $0.693/D$.

1.5.3 Population dynamics (competition, selection and mutation)

As mentioned in the previous section, bacteria growing under nutrient limitation are under selective pressure to change, in essence, to increase their specific growth-rate. Any cell increasing its specific growth-rate will have a competitive advantage, providing that the only interaction is for the limiting substrate and the environment remains constant. Indeed, a theoretical analysis of the growth of a contaminant or mutant arising within a 'steady state' continuous culture population (Powell, 1958), has concluded that the survival of such an organism not only depends on the relationship between the maximum specific growth-rates (μ_{\max}), and saturation constants (K_s), of the competing organisms, but also on the dilution rate at the time at

which a strain with a selective advantage arises.

Mutational changes affecting specific growth-rate, may therefore occur which are either selected for or against in the chemostat. Mutations may occur that do not affect specific growth-rate, and are therefore neutral mutations, in this context they do not afford a selectable phenotype. The appearance of neutral mutants within continuous culture populations has been shown to increase linearly with time (Novick & Szilard, 1950b). However, mutants that are selected against in the chemostat usually remain at a fixed proportion of the culture population with time, unless of course, the degree of selection against the mutant results in washout. However, fluctuations regularly occur in the proportion of neutral and disadvantaged mutants within a continuous culture. Such fluctuations appear to be the consequence of a selectable mutant arising within the culture population. Cells carrying a selectable mutation attain predominance within the culture population, over the neutral and disadvantaged mutant populations. Cells within this 'fitter' population, will again linearly accumulate neutral mutations with time. On the other hand, mutants that are selected against in the chemostat may decrease their fixed proportion within the population, depending on the selective advantage existing between the new 'fitter' and old disadvantaged strains (Novick & Szilard, 1950b). This phenomenon of regular fluctuations occurring in the proportions of neutral and disadvantaged mutants within continuous culture populations has been termed 'periodic

selection' (Atwood et al., 1951)

For spontaneous mutations, occurring usually at a frequency of 10^6 - 10^7 per cell, per generation for any one gene, the rate at which neutral mutants appear within continuous culture populations has been shown to be proportional to generation time, in both glucose- or phosphate-limited chemostat cultures (Kubitschek & Bendigkeit, 1964a). This indicates that spontaneous mutations occurring during chemostat cultures conform to the 'copy-error' hypothesis of mutation, in which spontaneous mutation rates, due to errors in DNA synthesis, would be expected to be proportional to the frequency of DNA replication and therefore generation time (Kubitschek, 1972).

In contrast to spontaneous mutations, analysis of increased mutation as a consequence of the action of mutagenic agents during continuous culture, has shown that such agents mediate mutation in one of two ways. Either mutagenesis occurs during replication of a gene (copy-error), and therefore the rate at which neutral mutants appear is proportional to the frequency of DNA replication, or as the result of mutagenesis occurring independently of DNA replication and therefore neutral mutants accumulate at a constant rate independent of generation time (Kubitschek & Bendigkeit, 1964b). Factors, therefore, that affect mutation rate can easily be distinguished by their dependence or lack of dependence on generation time in a 'steady state' culture.

Finally, one class of mutation that has been demonstrated to confer a selective advantage on the strain that carries it, is that of the mutator gene (Gibson *et al.*, 1970; Cox & Gibson, 1974). Mutator genes increase the spontaneous mutation rate of other genes (Painter, 1975). For *E.coli* K-12 the mutator genes mutH, mutL, mutS and mutT have all been shown to confer a selective advantage over an otherwise isogenic mut strain (Trobner & Piechocki, 1984). The mutT gene maps within a region of the *E.coli* chromosome, at 2 minutes, which contains a number of other genes that function to control cell division (Bachmann, 1983). mutT mediates an A-T to G-C transversion, and its function is dependent on DNA synthesis (Cox, 1976), suggesting that the mutT mutation may represent a defect in a gene whose product may be a component of the DNA replication machinery.

1.5.4 Plasmid stability

a) Introduction

Plasmids may exhibit instability as a consequence of either changes occurring in the integrity of the plasmid molecule, mainly resulting from the deletion or addition of genetic material, or from defects associated with plasmid maintenance functions, which would lead to the loss of an intact plasmid. The former instability is referred to as structural, while the latter as segregational instability.

It has been suggested that bacterial plasmids undergo alterations in their DNA structure as a

consequence of either micro- or macroevolutionary processes (Timmis et al., 1978). Microevolutionary changes may be defined as those changes occurring in DNA sequences that can be detected by the loss or gain of a restriction site, or by direct sequence analysis, and are assumed to occur primarily as a consequence of spontaneous mutagenesis. Macroevolutionary changes, on the other hand, may be defined as changes occurring in DNA sequences that can be detected by an alteration in the agarose gel electrophoresis pattern of endonuclease restricted and unrestricted plasmids, or by electron microscopic heteroduplex analysis. Such changes may involve either insertion, deletion or inversion of DNA segments (Cohen et al., 1978).

b) Structural instability

Plasmid genome rearrangements can occur independently of the recA gene product, and mainly involve site-specific recombination mediated by insertion sequences (IS), transposons (Tn), or by recombinogenic loci having properties analogous to sequences occurring within insertion sequences or transposable elements (i.e., inverted or direct repeat sequences), (Watanabe et al., 1982; Gomez-Eichelmann & Torres, 1983; Jones et al., 1982; Godwin & Slater, 1979; Kollek et al., 1980).

Inverted repeat sequences or palindromes are capable of forming stem and loop structures, which involves a change from the normal interstrand base pairing that occurs in the DNA duplex, to intrastrand base pairing

forming what is sometimes referred to as a 'hairpinned' or 'cruciform' structure. Cruciform structures have been proposed to occur in negatively supercoiled DNA, and experiments using S1 nuclease have shown that cruciform structures do occur in plasmids ColE1 and pBR322, as well as other covalently closed circular DNA molecules (Panayotatos & Wells, 1981; Lilley, 1980). Studies involving an inverted repeat sequence occurring within plasmid ColE1, have suggested that the kinetics for the formation of a cruciform structure may be dependent on such factors as temperature, ionic conditions, superhelical density and most significantly DNA base sequence (Lilley, 1985). Recently, a study of the physical and genetic properties of three palindromic sequences, which had been cloned into pBR322, showed that the two longer palindromes (146bp & 147bp), appeared to reduce the multimerization of pBR322, which to a certain extent normally occurs in wild-type recA strains of E.coli (Warren & Green, 1985). A several-fold increase in recA-dependent recombination was caused by the presence of the palindromic sequences in cis, although it was concluded that this property did not relate to the extent of cruciform structure formation. At a much lower frequency, recA-independent recombination was also observed to occur, however, these recombination events appeared to be analogous to recA-independent recombination normally observed between much longer palindromic sequences (usually greater than 800bp).

Two models have been proposed to account for deletion events occurring between repeat sequences (Albertini et al., 1982). The first model proposes that since inverted repeat sequences have the potential to form stem and loop structures, then during DNA replication, single-stranded DNA that is looped out from the template strand may be bypassed by the DNA synthesizing complex and therefore fail to be replicated. This form of aberrant DNA replication event has been termed 'slipped mispairing'. The second model proposes that recombination occurs between inverted or direct repeats, and that these events can be mediated independently of the recA gene product by enzymes that recognize such short homologous sequences. Support for this latter model comes from evidence which indicates that precise excision of transposable elements can be enhanced in certain E.coli K-12 mutants. For instance, mutations designated texA, occurring in the recB and recC genes, stimulate excision of transposons Tn5 and Tn10 (Lundblad et al., 1984). These mutant alleles apparently alter but do not abolish the RecBC function. Precise excision of Tn5, Tn10 and IS elements, has also been shown to occur following mutation in the uup locus, which maps at 21.3 minutes on the E.coli chromosome (Hopkins et al., 1983).

Mutant alleles occurring in the E.coli genome are not the only factors associated with precise excision of mobile elements. The F plasmid encodes ferA and ferB, that are involved in the precise excision of Tn5 and Tn10 (Hopkins et al., 1980). Mutations occurring in

ferA appear to eliminate excision, while mutations in ferB enhance the frequency of excision. In addition, ferB mutations increase recA-dependent recombination occurring between IS3 elements. This latter recombination event appears to be dependent on FerA, and may indicate involvement of FerA and FerB in integration of the F sex factor into the E.coli chromosome, via site-specific recombination between IS3 elements, postulated as being one class of fre site at which F plasmid integration occurs (Bresler et al., 1981).

c) Segregational instability

Competition between plasmid-free cells, following segregation during chemostat culture, and that of the plasmid-bearing population may be viewed as being analogous to the spontaneous generation of a mutant within the culture population (Powell, 1958). Essentially, the most important factors affecting the kinetics of appearance of plasmid-free cells would be the spontaneous rate at which they segregate, and the difference in the specific growth-rate between plasmid-free and plasmid-bearing populations (Kim & Ryu, 1984). Consideration of these two factors distinguishes the theoretical possibility of three situations arising as a consequence of the segregation of plasmid-free cells (Walmsley et al., 1983). These situations depend on whether, a) the plasmid within the plasmid-bearing population is selectively neutral or not, therefore, if selectively neutral plasmid-free cells would be expected to accumulate in a manner dependent on the rate of

Figure 1.32

Graphical representation of the theoretical kinetics of accumulation of plasmid-free cells during chemostat culture. The curves are generated from the following equations, and it is assumed that plasmid-bearing cells are either:

a) selectively neutral;

$$f = 1 - (1 - f_0) (1 - M)^t$$

b) at a selective disadvantage;

$$f = \frac{f_0 + \frac{M}{S} - \frac{M}{S} (1 - f_0) e^{-St}}{f_0 + \frac{M}{S} + (1 - f_0) e^{-St}}$$

c) at a selective advantage;

$$f = \frac{M}{S} - \frac{M}{S} - f_0 e^{-St} / t$$

where f = concentration of plasmid-free cells

f_0 = concentration of plasmid-free cells at commencement of continuous culture.

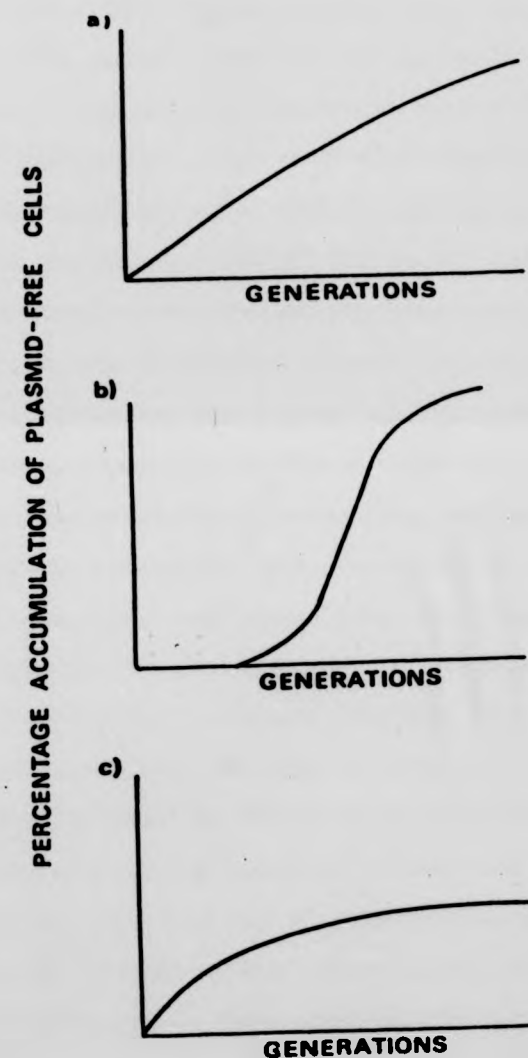
t_0 = time at commencement of chemostat culture

t = time or generations elapsed since t_0

M = mutation (segregation) rate

S = selection pressure = (growth-rate of parent) - (growth-rate of mutant).

(Reproduced from Walmsley *et al.*, 1983).



segregation of plasmid-free cells (Fig.1.32a), b) the plasmid-bearing population is at a selective disadvantage, then plasmid-free cells would be expected to accumulate to all intents and purposes exponentially (Fig.1.32b), and c) the plasmid-bearing population has a selective advantage, then the accumulation of plasmid-free cells would be expected to attain an equilibrium within the chemostat population consistent with the rate at which plasmid-free cells are generated and their washout from the chemostat vessel (Fig.1.32c).

Observations on the behaviour of plasmid-bearing cells during chemostat culture, have been made with respect to segregational stability and competition between plasmid-bearing and plasmid-free cells. Studies such as these have indicated that the fate of a plasmid-bearing strain depends on the plasmid, its host, nutrient-limitation, temperature and dilution rate. Plasmids that do not exhibit structural instability may be inherited either as the consequence of an active, or passive segregation process. In the case of passive inheritance, the kinetics of appearance of plasmid-free cells during 'steady state' continuous culture would be expected to depend on such parameters as plasmid copy number, cell shape and population density.

The most important parameter appears to be that of plasmid copy number, which may explain why derivatives of pBR322 and pACYC184, possessing alterations that prevent transcriptional interference with plasmid replication, are more stably maintained than the parent plasmid during phosphate-limited chemostat culture

(Vernet *et al.*, 1985). Likewise, plasmids such as pBR327 and pBR328, which contain deletions that remove transcriptional terminators in the terminal region of the tetracycline resistance gene, exhibit a greater degree of instability during continuous culture as a consequence of enhanced transcriptional interference with plasmid replication (Jones & Melling, 1984). Differences which may occur in the extent of superhelical density in different host backgrounds, may likewise affect the number of plasmid copies available for segregation at cell division, by altering the degree of transcriptional interference. However, a more important consideration with respect to host background may be the extent to which recombination events occur. This latter difference may explain why plasmid pAT153 is more stably maintained during chemostat culture in a strain that is recA, rather than in a wild-type recA background (Jones & Melling, 1984).

Differential effects of plasmid, host background, and culture conditions, on plasmid persistence during chemostat culture, have been demonstrated for plasmids pBR322 and pBR325 during glucose and nitrogen limitation in E.coli K-12 strains GY2354 and GM31 (Noak *et al.*, 1981). Whereas pBR322 was stably maintained under all growth conditions tested, in contrast to previously published findings (Jones *et al.*, 1980b; Jones & Melling, 1984; Vernet *et al.*, 1985; Wouters *et al.*, 1980), pBR325 free-cells preferentially segregated at a low dilution rate during glucose limitation. The preferential segregation of plasmid-free cells at low

dilution rates has also been shown for pBR322-bearing cells of a prototrophic E.coli strain, during glucose and phosphate limitation (Wouters et al., 1980). In addition, segregation of plasmid-free cells has also been shown to increase with an increase in the temperature of the culture. In all continuous culture experiments where the segregation of plasmid-free cells occurs, phosphate limitation has been shown to exert a greater selective pressure for the appearance of plasmid-free cells than other nutrient-limiting conditions. It has been suggested that phosphate-limitation exacts a greater stringency on DNA replication than does glucose limitation, and that nitrogen limitation may only be expected to influence or limit protein synthesis (Noak et al., 1981).

Continuous culture competition experiments between isogenic plasmid-free cells and cells bearing a stably inherited plasmid, have likewise indicated the importance of nutrient limitation on competition kinetics during phosphate-limited chemostat culture. For instance, growth of plasmid RPl-bearing cells in competition with isogenic plasmid-free cells is adversely affected by competition for the limiting nutrient (Melling et al., 1977), resulting in the predominance of plasmid-free cells over the RPl-carrying population (Fig.1.33). Batch culture experiments, using low concentrations of phosphate, have shown that the specific growth-rate of an isogenic plasmid-free cell is about twice that of RPl-bearing cells (Klemperer et al., 1979). In contrast to phosphate-limiting conditions,

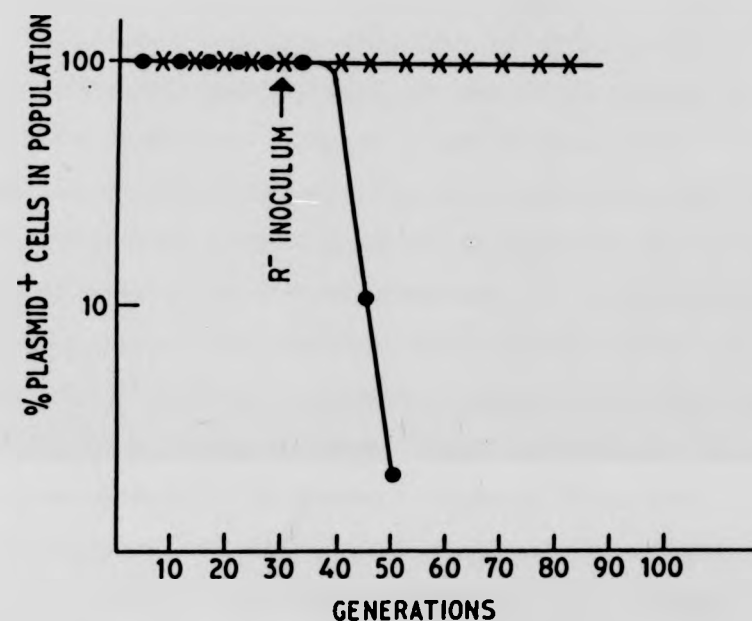


Figure 1.33

Persistence of plasmid RPl in *E.coli* strain W3110 during phosphate-limited chemostat culture. Maintenance of RPl in the absence of a plasmid-free host cell inoculum; x—x, accumulation of plasmid-free cells on addition of a 1% inoculum of isogenic plasmid-free cells; ●—●. (Reproduced from Primrose *et al.*, 1984).

similar kinetics of competition between plasmid-free and RPl-bearing cells were not observed during glucose-limited chemostat culture (Melling *et al.*, 1977). Results indicated the absence of any significant growth-rate advantage between plasmid-free and RPl-bearing cells. Competition for phosphate, as opposed to glucose, may limit the frequency at which plasmid RPl can initiate replication. A reduction in unit-copy plasmid replication, may as shown for the F plasmid (Ogura & Hiraga, 1983b), lead to cell division inhibition as a consequence of coupling between plasmid replication and cell division. Indeed, loss of RPl-bearing cells during phosphate-limited culture exhibited kinetics similar to those of theoretical 'wash-out' (Fig.1.33), suggesting that a halt had occurred in the cell division process.

Similar competition experiments to those carried out with plasmid RPl using both phosphate- and glucose-limited chemostat cultures, has demonstrated the competitive advantage of plasmid-free cells when in competition with isogenic cells carrying the stably inherited R6 plasmid (Wouters & van Andel, 1983). However, when nitrogen-limiting conditions were used, R6-bearing cells were found to have a selective advantage over the plasmid-free cells. The basis of this plasmid conferred selective advantage, during nitrogen-limited growth, is at present unknown. However, the carriage of accessory DNA elements, such as plasmids, lysogenic phage and insertion sequences, has in some instances been shown to confer a selective

advantage on the cells that bear them (Dykhuizen & Hartl, 1983).

1.5.5 Genetic elements that confer a selective advantage

Accessory DNA elements, which generally encode genes not essential to the host bacterium, have been defined as those DNA entities that are able to either replicate autonomously, or over-replicate their own DNA relative to that of the host chromosome (Campbell, 1981).

Accessory DNA elements have been postulated to have an evolutionary significance, in that they may provide an accessory gene pool which expands the normal or chromosomal gene pool of bacterial species. Since accessory DNA elements undergo interstrain or interspecies transfer, genes within the accessory gene pool could be supported and maintained within a small proportion of a population, without significant burden on the total bacterial population. Mechanisms which ensure the persistence within bacterial populations of accessory DNA elements are, therefore, of fundamental importance (Dykhuizen & Hartl, 1983).

Many plasmids have been shown to alter cellular functions, particularly with respect to DNA repair, mutagenesis, replication and recombination (Chernin & Mikoyan, 1981). For instance, plasmid pKM101 is capable of enhancing the mutation frequency of cells harbouring this plasmid (Langer *et al.*, 1981), pKM101 may therefore confer a selective advantage as a consequence of an increase in the frequency at which 'fitness' mutations occur, in a manner analogous to the selective advantage

conferred by mutator genes. Alternatively, a selective advantage may be conferred for a limiting nutrient by a seemingly unrelated plasmid function. This has been suggested recently by a study which showed that the tetracycline resistance gene of pBR322, mediates potassium transport in E.coli K-12 (Dosch et al., 1984).

In addition to possible plasmid functions conferring a selective advantage during nutrient limitation, it has been reported that E.coli lysogens of either P1, P2, Mu (Edlin et al., 1977), or lambda (Lin et al., 1977), have a selective advantage over nonlysogens during glucose-limited chemostat culture. The selective advantage of lambda lysogens has been attributed to the lambda rex gene function, and would appear to be specifically associated with the rexA gene product (Dykhuizen & Hartl, 1983). A similar selective advantage during glucose limitation has also been found to occur in E.coli cells harbouring the insertion element IS50 (Hartl et al., 1983). This conferred selective advantage did not depend on either the position at which the element inserted, or subsequent transposition, but was assumed to be the consequence of a direct physiological effect on the host cell. The IS50 insertion element encodes two functions, a transposase which catalyses transposition of the element, and a product that inhibits transposition. The topoisomerase-like characteristic of certain transposase enzymes was suggested as being responsible for the physiological change occurring within the host cell. When the copy number of IS50 was small, then the growth-rate advantage

was near proportional to the dosage of the IS50 element. However, the presence of the element on a high-copy number plasmid was found to adversely affect growth-rate. A similar beneficial growth-rate effect to that of IS50 has been shown to be conferred by the transposon Tn10 (Chao *et al.*, 1983). However, the basis of selective advantage in this case appears to involve transposition of the flanking IS10 elements, suggesting that the selective advantage may be the result of an increase in the frequency with which 'fitness' mutations occur, analogous to the selective advantage conferred by mutator genes.

1.5.6 Phosphate transport in E.coli K-12

As alluded to above, previous studies on the stability of plasmids during chemostat culture have in general demonstrated that phosphate-limited growth exerts a greater selective pressure for the appearance of plasmid-free cells. It was therefore decided that during the course of this study, all chemostat experiments where possible, would be conducted using phosphate-limited conditions.

The transport into E.coli K-12 of inorganic phosphate is mediated by two distinct systems, a low-affinity inorganic phosphate transport system (Pit), and a high-affinity phosphate-specific transport system (Pst), (Willsky & Malamy, 1980). The Pst system, during phosphate-limited chemostat culture, is derepressed by phosphate concentrations below 10 μ M (Saier 1979), whereas the Pit system is normally constitutive.

However, the intra- and extracellular exchange of inorganic phosphate mediated by the Pit system has, in the presence of glucose, been shown to be repressed during batch culture (Rosenberg *et al.*, 1982).

The Pst system appears to rely on the expression of a number of cell envelope components, the function of which is to scavenge inorganic phosphate and phosphate containing nutrients from the surrounding medium (Korteland *et al.*, 1982). Among these envelope components, the expression of which is regulated by limiting concentrations of phosphate, is the periplasmic protein for binding inorganic phosphate PhoS, the UgpB protein that binds glycerol-3-phosphate, alkaline phosphatase PhoA, and cytoplasmic membrane proteins involved as carriers in the transport of either inorganic phosphate (the products of the *pst* genes), or glycerol-3-phosphate (the *ugpA* gene product).

Studies in recent years have begun to unravel the complex genetic regulation of the Pst system. Most studies have concentrated on the expression of the *phoA* gene product. During derepression up to 6% of the total cell protein can be alkaline phosphatase (Saier, 1979). *phoA* gene expression appears to be positively regulated by the *phoB* gene product (Makino *et al.*, 1982), which in turn is itself positively regulated by the *phoR* and *phoM* gene products and negatively regulated by the *phoR*, *phoS*, *phoT* and *phoU* gene products (Shinagawa *et al.*, 1983). The *phoU* gene is located at the end of a cluster of genes which specify the *phoS*, *pstC*, *pstA* (*phoT*) and *pstB* gene products, all involved in the specific

transport of phosphate (Surin et al., 1985; Nakata et al., 1984). This cluster of genes is unidirectionally transcribed, they are all co-ordinately induced by phosphate limitation and may be arranged into an operon (Surin et al., 1985). In addition, they may be under the same genetic control as that of the phoA gene. Both the phoS and phoT gene products are involved in the transport of inorganic phosphate, and since mutations in either of these genes results in the constitutive expression of alkaline phosphatase, they may indirectly affect the regulation of the phoB gene product (Nakata et al., 1984). The product of the phoU gene, and those of the pstC and pstB genes, have been shown to be peripherally associated with the cytoplasmic membrane (Surin et al., 1985).

Finally, in addition to the phoA gene, it has been suggested that there are at least 18 unlinked phosphate starvation inducible promoters (psi) in E.coli K-12 (Wanner, 1983). One of the most well characterized of these phosphate regulated promoters is that for the gene encoding the outer membrane pore protein PhoE. The PhoE protein is involved in the formation of aqueous pores in the outer membrane, through which the uptake of phosphate and phosphate-containing compounds occurs (Tommassen & Lugtenberg, 1982). Expression of the phoE gene appears to be controlled in a similar manner to that of the phoA gene (Tommassen et al., 1984). However, the regulation of other phosphate starvation inducible promoters within the pho regulon appears to be only partially controlled. For instance, evidence

suggests that the hima gene, in addition to being a component of the SOS regulon, may also be induced by phosphate limitation (Wanner, 1983). Furthermore, expression of one particular phosphate starvation inducible promoter, the psi0 promoter, has been shown to be affected by mutations in the lon gene. Curiously, this lon-dependent induction of the psi0 promoter fused to lacZ, is in addition dependent on cell density (Wanner, 1983). Unfortunately, little other information is currently available concerning the nature or regulation of these, or other phosphate-starvation inducible promoters.

1.6 Aims of research

The plasmid cloning vector, pBR322, had previously been shown to be structurally stable but segregationally unstable during antibiotic-free chemostat culture (Jones et al., 1980b). The fundamental aim of this study was to define conditions under which pBR322 could be stably maintained during chemostat culture. Two approaches were considered. The first of these used the principle of auxotrophic complementation, while the second approach used naturally occurring plasmid-encoded stability functions. In conjunction with this second approach, studies were also initiated which were aimed at establishing whether plasmid ColE1 encoded a partitioning function, that had previously been postulated by Jones et al., (1980b). Although the immediate aim of this study was the establishment of a strategy by which pBR322 could be stably maintained, it quickly became obvious that initial concepts, established by

Jones et al. (1980b), regarding plasmid maintenance during chemostat culture were limited, and that a more complicated picture began to emerge with respect to plasmid-host interactions and plasmid maintenance. An interesting development to occur was the isolation of host mutant plasmid-free segregants. This resulted in re-direction of the aims of research towards determining the phenotypic characteristics of these host mutants, with a view to establishing subsequent studies aimed at a genetic characterization of the mutant strains.

CHAPTER II

MATERIALS AND METHODS

2.1 Reagents

Unless otherwise stated, reagents used routinely were of Analar grade obtained from British Drug Houses (BDH) Chemicals Limited, Poole, England. With a few stated exceptions, all amino acids, vitamins, antibiotics and other media supplements were obtained from Sigma Chemical Co. Ltd., Poole, England.

2.2 Sterilization of media and buffers

Sterilization was routinely carried out by either autoclaving at 15psi (121°C) for 20 minutes, or passing a solution through a 0.45µm Millipore filter (Millipore (U.K.) Limited, 11-15 Peterborough Road, Harrow, Middlesex, England).

2.3 Bacterial strains

Bacterial strains employed during the course of this study are listed in table 2.1.

2.4 Storage and maintenance of bacterial strains

Bacterial strains were stored at -20°C as 1ml suspensions in sterile 0.1M potassium phosphate buffer pH7.0 containing 20% (v/v) glycerol. When revived strains were maintained on L-agar in an air-tight box at 4°C.

2.5 Batch culture media

Batch culture growth was carried out in either rich medium, formulated as LB-medium (L-broth or L-agar), (Miller, 1972a), or minimal medium, formulated as A+B

Table 2.1: Bacterial strains

Designation	Parent strain	Relevant genotype	Other characteristics	Source
CB100 (=PA340)		<u>gdh</u> -1, <u>gluB</u> 31, <u>str</u> -9	F ⁻ , <u>thr</u> -1, <u>leu</u> -6 <u>thi</u> -1, <u>lacY</u> -1, <u>mal</u> 1, <u>xy</u> 1-7, <u>ara</u> -13, <u>mt</u> 1-2, <u>ton</u> A2, <u>gal</u> -6, <u>his</u> -1, <u>arg</u> H1, <u>sup</u> E44, <u>lambda</u> R, <u>lambda</u> -	ICI Corporate Laboratory, Runcorn, Cheshire. (Windass et al., 1980)
JW114	CB100 carrying pACYC184/ <u>gdh</u> ⁺	"	"	"
WX100	CB100	<u>gdh</u> -1 <u>gluB</u> 31, <u>str</u> -9, <u>recA</u> 56, <u>slr</u> C300::Tn10	As CB100	P. Derbyshire. (This study).
W5445	C600 (=CR34)	<u>str</u> ^R , <u>hsd</u> R, <u>hsd</u> M, (<u>r</u> -k m ⁻ k)	F ⁻ , <u>thr</u> -1, <u>leu</u> -6 <u>pro</u> , <u>thi</u> -1, <u>lacY</u> -1, <u>ton</u> A21, <u>sup</u> E44, <u>lambda</u> -	S.D. Ehrlich, Institut de Recherche en Biologie Moléculaire, Paris. (Jones & Primrose et al., 1980).
WX9-2	W5445	As W5445 (Mutant plasmid-free segregant)	As W5445	P. Derbyshire. (This study).
WX11-2	"	"	"	"
WX11-3	"	"	"	"
WX11-8	"	"	"	"

Designation	Parent strain	Relevant genotype	Other characteristics	Source
HB101		<u>str</u> -20, <u>recA</u> 13, <u>hsd</u> S20 (<u>r</u> ⁻ B ^m B)	F ⁻ , <u>pro</u> A2, <u>lacY</u> 1, <u>xy</u> 1-5, <u>ara</u> -14, <u>mt</u> 1-1, <u>gal</u> K2, <u>sup</u> E44, <u>lambda</u> -	N.H. Mann, University of Warwick, Coventry. (Boyer & Roulland-Dussoix, 1969).
X156	W945	<u>str</u> -109, <u>azi</u> -6	F ⁻ , <u>leu</u> -6, <u>pro</u> C34, <u>pur</u> E42, <u>trp</u> E38, <u>thi</u> -1, <u>ara</u> -14, <u>lacY</u> 1, <u>gal</u> K2, <u>xy</u> 1-5, <u>mt</u> 1-1 <u>ton</u> A23, <u>tsx</u> -67, <u>sup</u> E44, <u>lambda</u> -	A. Markovitz, University of Chicago. (Berg et al., 1976).
RC103	X156	<u>str</u> -109, <u>azi</u> -6, <u>cap</u> 89	As X156 but <u>pro</u> ⁺	A. Markovitz, University of Chicago. (Gayda et al., 1976).
AB1157	W945	<u>str</u> -31	F ⁻ , <u>thr</u> -1, <u>leu</u> -6, <u>pro</u> A2, <u>thi</u> -1, <u>ara</u> -14, <u>lacY</u> 1, <u>gal</u> K2, <u>xy</u> 1-5, <u>mt</u> 1-2, <u>tsx</u> -33, <u>arg</u> E3, <u>his</u> -4, <u>sup</u> E44, <u>sup</u> 37 <u>lambda</u> -	J. Hinton, University of Warwick, Coventry. (Johnson, 1977).
PAM660	AB1157	<u>str</u> -31, <u>lon</u> -22	As AB1157 but <u>arg</u> ⁺ , <u>met</u>	C. Jones, University of Leicester. (Johnson, 1977).
PAM161	PAM660	<u>str</u> -31, <u>lon</u> -22, <u>sul</u> B-25	"	S.B. Primrose, G.D. Searle & Co. Ltd., High Wycombe. (Johnson, 1977).
PAM163	"	<u>str</u> -31, <u>lon</u> -22, <u>sul</u> A-27	"	"

Table 2.1 Con't.:

<u>Designation</u>	<u>Parent strain</u>	<u>Relevant genotype</u>	<u>Other characteristics</u>	<u>Source</u>
Ymel			F ⁺ , mel-1, supE57, supF58, (lambda).	S.B. Primrose, University of Warwick, Coventry. (Bachmann, 1972).
JC10240		recA56, srlC300::Tn10 (P1cmclr100)		S.B. Primrose, University of Warwick, Coventry.
HW27	AB1899	lon-1	Segregates out nonmucoid colonies	S.B. Primrose, G.D. Searle Limited, High Wycombe, U.K.

were added to sterile minimal medium to give a final concentration of 0.2% (w/v). Required amino acids, which had been filter-sterilized, were added to minimal medium to give a final concentration of 50µg/ml.

2.6 Determination of glutamate dependence

The dependence of E.coli strains on glutamate for growth was determined by incubating cells overnight at 37°C on A+B minimal agar, containing appropriate amino acids and vitamins with and without 2mg/ml L-glutamate (Sigma Chemical Co.Ltd.).

2.7 Preparation of antibiotic solutions

Filter-sterilized antibiotics were prepared in the following manner and added to solid or liquid media at the following final concentrations:

<u>Ampicillin</u>	Prepared as a 10mg/ml stock solution
<u>sodium salt:</u>	in distilled H ₂ O and stored at -20°C, final concentration 100µg/ml.
<u>Tetracycline</u>	Prepared as a 1mg/ml stock solution in
<u>HCl:</u>	distilled H ₂ O and stored at -20°C, final concentration 10µg/ml.
<u>Streptomycin</u>	Prepared as a 5mg/ml stock solution in
<u>sulphate:</u>	distilled H ₂ O and stored at -20°C, final concentration 50µg/ml.

- Chloramphenicol: Prepared fresh as a 10mg/ml stock solution, in either methanol or ethanol, and used at a final concentration of 100µg/ml for plasmid DNA amplification, or 12.5µg/ml for selecting Plcm lysogens.
- Nitrofurantoin: Prepared fresh as a 0.4mg/ml stock solution in distilled H₂O, final concentration 4µg/ml.
- Nalidixic acid sodium salt: Prepared fresh as a 5mg/ml stock solution in 0.1M NaOH, final concentration 50µg/ml.
- Oxolinic acid: Prepared fresh as a 0.05mg/ml stock solution in 0.1M NaOH, final concentration 0.5µg/ml.
- Novobiocin sodium salt: Prepared fresh as a 50mg/ml stock solution in distilled H₂O, final concentration 500µg/ml.
- Coumermycin A₁: Prepared fresh as a 2mg/ml stock solution in dimethyl sulphoxide, final concentration 20µg/ml.
- Sodium azide: Prepared fresh as a 10mg/ml stock solution in distilled H₂O, final concentration 100µg/ml.
(BDH Chemicals Limited)
- Methyl methane-sulphonate: Supplied as a liquid and used at a final concentration of 250µl per litre.
(Aldrich Chemical Co.Ltd.,
Gillingham,
England).

2.8 Determination of antibiotic sensitivity

The sensitivity of E.coli strains to the antimicrobial compounds methyl methanesulphonate, nitrofurantoin, sodium azide, nalidixic acid, oxolinic acid, novobiocin and coumermycin A₁, were scored by streaking cells of the strain to be tested onto antibiotic-free and antibiotic-containing L-agar, at the stated final concentration (see Section 2.7). The inoculated plates were incubated overnight at an appropriate temperature, and growth compared on the antibiotic-free and antibiotic-containing medium.

2.9 Preparation of post U.V.-light irradiation plating medium containing DL-pantoyllactone

DL-pantoyllactone was prepared as a fresh 1.3g/ml stock solution in distilled H₂O and following sterilization by autoclaving, added to L-agar to give a final concentration of 13mg/ml.

2.10 Serial dilutions of bacterial cells

These were carried out using a 0.9% (w/v) solution of NaCl in distilled H₂O (isotonic saline), sterilized by autoclaving.

2.11 Centrifugation

Centrifugations were carried out using equipment supplied by MSE Scientific Instruments, Crawley, West Sussex, England.

2.12 Spectrophotometry

Measurements of optical density at appropriate wavelengths, were carried out using an SP600 series 2 visible spectrophotometer (Pye Unicam Ltd., York St., Cambridge, England).

2.13 Phase-contrast microscopy of bacterial cells

A drop of isotonic saline was placed onto a glass slide, bacterial cells were transferred to and dispersed in the drop by the stirring action of a loop. A glass cover slip was placed over the suspension, and cells were viewed under phase-contrast (1,000-fold magnification), using a Gillett and Sibert Ltd., microscope.

2.14 Scanning electron microscopy of bacterial cells

Detailed cellular morphology of bacterial cells was examined by scanning electron microscopy, cells were prepared by D. Wood of the National Institute of Biological Standards and Control, Holly Hill, Hampstead, London, England, in the following manner:

- 1) Cells were cultured at 37°C in 10ml minimal medium, until an OD_{650nm} of 0.6. The culture was then shifted to 42°C and incubation continued for a further 2hrs.
- 2) Cells were harvested by centrifugation (MSE Chilspin, 6 x universal-angled rotor), at 5,000rpm for 10mins, washed in isotonic 1M sodium cacodylate buffer pH7.4 (cacodylate buffer), and repelleted.

- 3) The final pellet was resuspended in 200 μ l 2.5% (w/v) glutaraldehyde in cacodylate buffer, so as to fix the cells.
- 4) One drop of a suspension of the fixed cells, was placed onto a polylysine treated glass coverslip for 4-5mins, to allow the cells to adhere to the glass surface (Mazia et al., 1975).
- 5) The coverslips were then washed with cacodylate buffer, and post-treated with 1% OsO₄ in cacodylate buffer for 1hr.
- 6) The coverslips were then rinsed in cacodylate buffer, and treated with tannic acid for 20mins. (Katsumoto et al., 1981).
- 7) The coverslips were again washed in cacodylate buffer, and the cells were dehydrated through a graded series of ethyl alcohol, they were then critical point dried in liquid CO₂ using a Polaron critical point dryer.
- 8) The dried coverslips were mounted onto SEM studs and sputter coated with 7nm of gold/palladium.
- 9) The cells were examined using a Philips Series 501, scanning electron microscope.
- 10) The scanning electron image was photographed onto Ilford FP4 ASA125 roll film, and developed negatives were enlarged 3-fold on resin coated glossy paper, giving final magnifications of 10,000-, 20,000- or 40,000-fold.

2.15 Determination of the plating efficiency on rich medium of *E.coli* strains grown in minimal medium

The plating efficiency on rich medium, of bacterial strains grown in minimal medium, was determined essentially as described by Berg et al. (1976) in the following manner:

- 1) Cells were grown overnight at 37°C in A+B minimal medium, with appropriate supplements of amino acids and vitamins.
- 2) Cells were chilled on ice for 5mins.
- 3) Serial dilutions were made into isotonic saline solution to 10^{-7} .
- 4) 0.1ml aliquots from each serial dilution were spread on A+B minimal agar (containing appropriate amino acids and vitamins), and L-agar. The plates were incubated overnight at 37°C.
- 5) Viable counts on A+B minimal agar and L-agar were determined.
- 6) Plating efficiency was calculated as the ratio of the average of the viable counts on L-agar, and A+B minimal agar from two separate platings.

2.16 Determination of the sensitivity of *E.coli* strains to U.V.-light irradiation

The killing kinetics of bacterial strains exposed to U.V.-light irradiation was determined using the basic method of Miller, (1972b) and carried out in the following manner:

- 1) Cells were grown in 10ml L-broth either overnight or to an OD_{650nm} of 0.6.
- 2) Cells were harvested by centrifugation (MSE Chilspin, 6 x universal-angled rotor), at 5,000rpm for 10mins, and the pellet was resuspended in 10ml isotonic saline solution.
- 3) 0.2ml of cells were transferred to a 1.5ml Eppendorf tube and placed on ice. (Viable count determination of this sample indicated survival of cells not exposed to U.V.-light irradiation.)
- 4) The remainder of the resuspended culture was decanted into a 10cm diameter glass Petri dish, which had been sterilized by autoclaving.
- 5) The U.V.-light source, located in a dimly lit area of the laboratory, was a CAMG, Universal U.V. lamp set at 254nm (Camlab Ltd., Cambridge, England). The lamp was allowed to warm up for at least 30mins before use, and calibrated to 0.4 Joules m⁻²sec⁻¹, by the use of a 'Blakray' U.V. dosimeter (Ultra Violet Products, Inc., San Gabriel, California, USA).
- 6) With the glass lid securely placed over the Petri dish, the dish was placed below the U.V.-light source.
- 7) The glass lid was removed to allow U.V. irradiation of the cells for a set time interval. The Petri dish was gently rotated during each period of U.V.-light exposure.
- 8) At the end of each period of U.V.-light irradiation, a 0.2ml sample of cells was transferred to a 1.5ml Eppendorf tube and placed on ice.

9) Suitable dilutions in isotonic saline solution were carried out, and 0.1ml of each dilution was spread on appropriate L-agar medium to determine the number of viable cells remaining.

10) The logarithm of the percentage of cells surviving was plotted against the fluence of U.V.-light in Joules m^{-2} .

2.17 Construction of *E.coli* *recA* derivatives using P1-mediated transduction

Construction of recombination deficient cells, was mediated by bacteriophage P1 transduction of the *recA56* allele of *E.coli* K-12 strain JC10240. This strain has the following relevant genotype, *recA56*, *sr1C300::Tn10*. Bacteriophage P1 was grown on this strain and used to transduce recipient cells to tetracycline resistance (i.e. *Tn10*). Over 80% of transductants should also have acquired the *recA56* locus. The following protocol was used:

- 1) Strain JC10240 (*P1cm~~c~~lr100*), was grown in 10ml L-broth + 0.01M $MgSO_4$ at 32°C with shaking until OD_{650nm} was about 0.6.
- 2) The culture was further incubated at 40°C for 35mins.
- 3) The cells were then incubated at 37°C for 90mins and the culture was treated with 0.2ml of $CHCl_3$, vortexed for about 2mins, and stored at 4°C overnight.
- 4) This crude lysate was centrifuged (MSE Chilspin, 6 x universal-angled rotor), at 5,000rpm for 15mins, and

the supernatant was carefully decanted into glass bijous containing a few drops of CHCl_3 and stored at 4°C .

5) The phage lysate was checked for sterility and titred at 42°C on E.coli K-12 strain Ymel.

6) Recipient cells were grown in 10ml L-broth + 0.01M MgSO_4 at 32°C to an $\text{OD}_{650\text{nm}}$ of 0.6.

7) 0.5ml aliquots of this culture were dispensed into 0.5ml L-broth + 0.01M MgSO_4 (control), and 0.5ml of the P1 lysate diluted in L-broth + 0.01M MgSO_4 to about 4×10^8 pfu/ml, and incubated at 32°C for 1hr.

8) The transduction mix and controls were spread on L-agar containing $10\mu\text{g/ml}$ tetracycline, and incubated at 37°C overnight. Control plates should show no growth.

9) Tetracycline-resistant transductants were transferred, using sterile toothpicks, to L-agar plates containing $10\mu\text{g/ml}$ tetracycline, and L-agar plates containing $4\mu\text{g/ml}$ nitrofurantoin. The plates were incubated overnight at 37°C .

10) Tetracycline-resistant colonies which are nitrofurantoin-sensitive, should have acquired the recA56 allele. This was confirmed by comparing the U.V.-light sensitivity of the transductants and the recipient parent strain. One half of an L-agar plate was seeded with a transductant, and the other half with the parent strain. Portions of the culture plate, exposing comparable areas inoculated with transductant and parent strain, were irradiated by U.V.-light for increasing periods of time. The plates were incubated at 37°C overnight, and growth of parent and transductant strains compared.

2.18 Plasmids

Plasmids constructed and employed during the course of this study are listed in table 2.2.

2.19 Storage of plasmid DNA

Plasmid DNA was stored as a solution in sterile 1mM EDTA, 10mM tris pH8.0 at 4°C.

2.20 Agarose gel electrophoresis of plasmid DNA

Plasmid DNA was analysed using agarose gel electrophoresis. Horizontal 1% (w/v) agarose, type 1: low EEO (Sigma Chemical Co. Ltd.), gels were prepared in tris-acetate buffer (0.02M sodium acetate, 2mM EDTA and 0.04M tris adjusted to pH8.3 with glacial acetic acid), and ethidium bromide was added to a final concentration of 5µg/ml. Samples were electrophoresed at a constant voltage of 70V for about 3hrs, and photographed under U.V.-light using Polaroid type 665 film and an orange filter.

2.21 Rapid, small-scale preparation of plasmid DNA from E.coli

For the routine analysis of plasmid DNA, the small-scale rapid isolation procedure as described by Holmes & Quigley, (1981) was performed in the following manner:

Table 2.2: Plasmids

<u>Designation</u>	<u>Derivation</u>	<u>Replication origin</u>	<u>Relevant properties</u>	<u>Other characteristics</u>	<u>Source</u>
pACYC184/ <u>gdh</u> ⁺		pl5A	<u>gdh</u> ⁺	Cm ^R , Tc ^S	ICI Corporate Laboratory, Runcorn, Cheshire. (Windass et al., 1980).
pWX15	7.6kb pACYC184/ <u>gdh</u> ⁺ <u>Sal</u> I fragment inserted at <u>Sal</u> I site pBR322	pBR322	<u>gdh</u> ⁺ , <u>mob</u> ⁺ <u>rop</u> ⁺	Ap ^R , Tc ^S	P. Derbyshire. (This study).
pWX9	<u>Eco</u> RI/ <u>Ava</u> I region of pWM31 substituting for the <u>Eco</u> RI/ <u>Ava</u> I Tc ^R coding region of pBR322	"	<u>par</u> ⁺ (pSC101), <u>mob</u> ⁺ , <u>rop</u> ⁺	"	I.M. Jones, University of Warwick, Coventry.
pWX9-8	Dimer of pWX9 isolated from strain WX11-8 (see table 2.1)	"	"	"	P. Derbyshire. (This study).
pWX9-161	Monomer of pWX9 isolated from strain PAM161 (see table 2.1)	"	"	"	"
pWX9-163	Dimer of pWX9 isolated from strain PAM163 (see table 2.1)	"	"	"	"

<u>Designation</u>	<u>Derivation</u>	<u>Replication origin</u>	<u>Relevant properties</u>	<u>Other characteristics</u>	<u>Source</u>
pWX11	BaeIIA fragment of pDS1109, inserted at an <u>Bae</u> II site within the Tc ^R coding region of pBR322	pBR322	<u>mob</u> ⁺ , <u>rop</u> ⁺	Ap ^R , Tc ^S , Iel	I.M. Jones, University of Warwick, Coventry.
pWX11-8	Dimer of pWX11 isolated from strain WX11-8 (see table 2.1)	"	"	"	P. Derbyshire. (This study).
pWX13	10.49kb (4.8kb + 5.69kb) lambda <u>Eco</u> RI fragment insert at <u>Eco</u> RI site pDS1109	ColE1	"	Ap ^R , Iel	"
pWX14	4.8kb lambda <u>Eco</u> RI fragment insert at <u>Eco</u> RI site pDS1109	"	"	"	"
pDS1109	ColE1::Tn1	"	"	"	D. Sherratt, University of Glasgow. (Dougan & Sherratt, 1977).

Table 2.2 Con't.

<u>Designation</u>	<u>Derivation</u>	<u>Replication origin</u>	<u>Relevant properties</u>	<u>Other characteristics</u>	<u>Source</u>
RSF2124	ColEI::Tn1	ColEI	<u>mob</u> ⁺ , <u>rop</u> ⁺	Ap ^R , Iel, <u>cea</u> ⁺	S.B. Primrose Warwick, Coventry. (So et al., 1975).
pNT3		"		Ap ^R	J. Tomizawa, National Institutes of Health, Bethesda, Maryland, USA. (Ohmori & Tomizawa, 1979).
pOU93		pBR322	<u>parB</u> ⁺ (RL), <u>mob</u> ⁺ , <u>rop</u> ⁺	Tc ^R , Ap ^S	K. Gerdes, University of Odense. (Gerdes et al., 1985).
pBR322	(see Figure 1.10)	"	<u>mob</u> ⁺ , <u>rop</u> ⁺	Ap ^R , Tc ^R	S.D. Ehrlich, Institute de Recherche en Biologie Moléculaire, Paris. (Bollivar et al., 1977).
pAT153		"	<u>mob</u> , <u>rop</u>	"	D. Sherratt, University of Glasgow. (Twigg & Sherratt, 1980).
pRM31		p15A	<u>par</u> ⁺ (pSC101)	Tc ^R	P. Meacock, University of Leicester. (Meacock & Cohen, 1980).

- 1) Plasmid-bearing cells were grown overnight at 37°C in a medium containing an appropriate selective antibiotic (either 10ml A+B minimal medium with casamino acids, or L-broth).
- 2) 1.5ml of this culture was transferred to a 1.5ml Eppendorf tube, and the cells were pelleted in an Eppendorf microcentrifuge for 20secs.
- 3) The pellet was resuspended, by vortexing in 25µl of the following lysis buffer;

Sucrose	-	8% (w/v)
Triton X-100	-	5% (v/v)
EDTA	-	50mM
Tris	-	50mM (pH8.0)

- 4) 4µl of a freshly prepared solution containing lysozyme (Sigma Chemical Co. Ltd., 10mg/ml lysozyme in 0.25M tris pH8.0), was then added to this suspension.
- 5) The mixture was vortexed and left at room temperature for 2-3mins.
- 6) The sample tube was then placed in a boiling water bath for 40-60secs.
- 7) The sample was removed and centrifuged in an Eppendorf microcentrifuge for 10mins.
- 8) The supernatant, about 50µl, was transferred to a fresh tube (the gelatinous pellet was discarded).

9) 10 μ l 5M ammonium acetate solution and an equal volume of isopropanol at -20°C, was then added to the supernatant. The mixture was vortexed, and nucleic acids precipitated at -70°C in a dry ice/ethanol bath for 10mins.

10) The precipitate was pelleted in an Eppendorf microcentrifuge for 10mins.

11) The supernatant was discarded and the tube carefully rinsed with 100 μ l of 80% (v/v) ethanol (-20°C), and microcentrifuged for 2mins.

12) The ethanol was discarded, and the pellet was vacuum dried.

13) The pellet was then resuspended in 10-20 μ l of 1mM EDTA, 10mM tris pH8.0, and analysed by agarose gel electrophoresis.

2.22 Large-scale preparation of plasmid DNA from E.coli

For the purification of plasmid DNA used in cloning procedures or transformations, a large-scale preparative method adapted from the methodologies of Clewell & Helinski, (1969) and Humphreys *et al.* (1975) was used:

1) Cells were grown at 37°C to an OD_{650nm} of 0.9, in either 500ml A+B minimal medium with casamino acids or L-broth, contained in a 2 litre bevelled flask to give maximum aeration. Plasmid content was amplified by the addition of 100 μ g/ml chloramphenicol and incubation was continued at 37°C overnight.

- 2) The cells were harvested by centrifugation at 5,000rpm (6 x 250ml angled rotor), for 10mins, and the pellet was resuspended in a 25ml solution containing 100mM NaCl, 3mM EDTA, 50mM tris pH8.0.
- 3) The cell suspension was transferred to an MSE 40ml screwtop centrifuge tube, and cells were pelleted at 5,000rpm (8 x 40ml angled rotor), for 10mins.
- 4) The pellet was resuspended in 7.5ml lysis buffer, containing 25% (w/v) sucrose in 50mM tris pH8.0, and placed on ice for 10mins.
- 5) 1.5ml of a freshly prepared lysozyme solution (Sigma Chemical Co.Ltd., 10mg/ml lysozyme in 0.25M tris pH8.0), was then added. The solution was gently mixed and placed on ice for 5mins.
- 6) 3ml 0.25M EDTA pH8.0 was then added, and the preparation gently mixed and placed on ice for a further 5mins.
- 7) Finally, 12ml of a solution of 1% (w/v) Brij 58 (Sigma Chemical Co.Ltd.), 0.4% (w/v) sodium deoxycholate, 2.5mM EDTA, 50mM tris pH8.0 was added and gently mixed. The mixture was placed on ice for 10mins. If sufficient clearing of the solution had not occurred within this time period, then the mixture was incubated at 37°C for 5-10mins.
- 8) The mixture was centrifuged at 18,000rpm (8 x 40ml angled rotor), for 45mins, at 10°C. The supernatant, about 20ml, was decanted into a fresh 40ml screwtop centrifuge tube.

9) Plasmid DNA was precipitated overnight at 4°C by the addition and gentle mixing of 5ml 5M NaCl, and 8ml 50% (w/v) polyethylene glycol 6000 in 10mM sodium phosphate pH7.0.

10) The precipitate was pelleted by centrifugation at 5,000rpm (8 x 40ml angled rotor), for 5mins.

11) The pellet was vacuum dried and resuspended in 5ml 50mM NaCl, 5mM EDTA, 50mM tris pH8.0.

12) 5.2g of CsCl was dissolved in the 5ml suspension, and 200µl of a solution of ethidium bromide was then added (Sigma Chemical Co.Ltd., 10mg/ml ethidium bromide in distilled H₂O). The dissolved CsCl solution was transferred to a 10ml MSE nitrocellulose ultra-centrifugation tube and a density gradient was allowed to form, by centrifugation at 36,000rpm in a titanium type 50 fixed angled rotor, for 60 hours at 18°C.

13) Plasmid DNA bands were detected by illumination with a long-wave U.V.-light source. Bands were harvested, from the top of the gradient first, by the use of a 2ml syringe and G19 needle, carefully lowered into the gradient material.

14) Ethidium bromide was extracted from isolated plasmid DNA bands by adding an equal volume of CsCl-saturated isopropanol (10ml of distilled H₂O saturated with CsCl, added to 250ml isopropanol, distilled H₂O was added to this solution until all the CsCl had dissolved).

15) The two immiscible solutions were mixed by inverting repeatedly. The upper pink layer was removed and discarded.

16) The extraction procedure was repeated until ethidium bromide was not detectable by U.V. illumination. Two further extractions were then carried out.

17) CsCl was removed from the plasmid DNA sample by overnight dialysis at 4°C, against 4 litre of 1mM EDTA, 10mM tris pH8.0.

2.23 Preparation of dialysis tubing

Dialysis tubing which was supplied by The Scientific Instrument Centre Ltd., 1 Leeke St., London, England, was treated before use in the following manner:

1) Tubing was first boiled for 15mins in 5% (w/v) sodium bicarbonate, this was repeated twice to remove U.V.-light adsorbing material.

2) The tubing was then boiled for 15mins in 1mM EDTA pH8.0, to remove metal ions.

3) Tubing was finally rinsed in distilled H₂O, and stored after sterilization by autoclaving in distilled H₂O at 4°C.

2.24 Ethanol precipitation of DNA

At various times it was necessary to concentrate or desalt DNA preparations, this was accomplished by ethanol precipitation:

- 1) 1/10 volume of 3M sodium acetate pH8.0, was added to a known volume of a solution containing DNA.
- 2) 2 volumes of absolute ethanol (-20°C) were then added, and in the case of, a) large volumes, the container was placed in a -70°C freezer for at least 2hrs., or b) small volumes, the container was placed in a dry ice/ethanol bath for 10mins.
- 3) The precipitated DNA was pelleted at either 18,000rpm (8 x 40ml angled rotor), for 10mins at -10°C for large volumes, or in an Eppendorf microcentrifuge for 10mins at room temperature for small volumes.
- 4) The DNA pellet was vacuum dried and resuspended in a suitable volume of a solution containing 1mM EDTA, 10mM tris pH8.0.

2.25 Phenol extraction of DNA

At various times it was necessary to remove contaminating protein material from DNA preparations, this was carried out by phenol extraction:

- 1) An equal volume of phenol deproteinizing mixture was added to a known volume of a solution containing DNA. Phenol deproteinizing mixture consisted of 100g of phenol dissolved in 100ml of chloroform, with 4ml of iso-amylalcohol and 0.1g 8-hydroxyquinoline added, this was stored under 10mM tris pH7.5 at 4°C .
- 2) The immiscible solutions were thoroughly mixed and separated by Eppendorf microcentrifugation for about 2mins.

3) The upper aqueous layer was removed to a fresh tube, and the phenol layer was extracted with an equal volume of a solution containing 1mM EDTA, 10mM tris pH8.0, the solutions were separated by Eppendorf microcentrifugation for about 2mins.

4) The aqueous phases from steps (2) and (3) were pooled, and the phenol removed by adding an equal volume of a 24:1 mixture of chloroform and iso-amylalcohol. The solutions were mixed and separated by Eppendorf microcentrifugation for about 2mins.

5) The upper aqueous phase was transferred to a fresh tube, and the DNA was ethanol precipitated as described above.

2.26 Restriction endonuclease digestion of DNA

Restriction analysis and cloning strategies for plasmid DNA, employed commercial preparations of restriction enzymes (Boehringer Co. (London) Ltd., Lewes, East Sussex), and were carried out under conditions recommended by the vendor.

2.27 Ligation of DNA fragments

Ligation of DNA fragments generated by restriction endonuclease digestion, were performed using T4 DNA ligase (Bethesda Research Laboratories (U.K.) Limited., Cambridge, England). Ligation reactions were carried out as follows:

- 1) 2 μ l of ligase buffer was added to 18 μ l of a solution containing DNA to be ligated. Ligase buffer was freshly prepared as follows: 1 μ l of stock bovine serum albumin (Sigma Chemical Co.Ltd., 0.01g/ml in distilled H₂O), 0.5 μ l of stock beta-mercaptoethanol (50% (v/v) in distilled H₂O), and 1 μ l of stock adenosine triphosphate (Sigma Chemical Co. Ltd., 0.25g/ml in distilled H₂O), were added to 18 μ l of sterile ligase salts base consisting of 12mM EDTA, 70mM MgCl₂ and 300mM tris pH7.6.
- 2) 0.05 units or 0.2 units of T4 DNA ligase, for either cohesive-end or blunt-end ligations respectively, were added to each 20 μ l reaction mix.
- 3) The ligation mixture was incubated at 15°C overnight.
- 4) Ligation was checked by agarose gel electrophoresis, and the ligated DNA was used to transform the appropriate E.coli K-12 strain.

2.28 Transformation of E.coli with plasmid DNA

Plasmid-bearing strains were generated by transformation, and stored as described in Section 2.4. The transformation procedure was based on the methodology described by Humphreys et al. (1979) and carried out as follows:

- 1) 10ml of L-broth was inoculated with one colony from an L-agar plate of the bacterial strain to be transformed, and incubated at 37°C with shaking until OD_{650nm} was between 0.6 and 0.9.

- 2) The cells were centrifuged at 5,000rpm (MSE Chilspin, 6 x universal-angled rotor), for 10mins and the pellet was resuspended in 5ml of ice cold CaCl_2 (0.05M CaCl_2 (Calcium chloride dihydrate Grade 1, Sigma Chemical Co.Ltd.,) in 10mM tris pH8.0), and placed on ice for 20mins.
- 3) Cells were centrifuged at 5,000rpm (MSE Chilspin, 6 x universal-angled rotor), for 10mins and resuspended in 1ml of ice cold CaCl_2 (0.05M), these competent cells were stored on ice until required.
- 4) For transformation, 10 μ l of a solution containing plasmid DNA was added to 200 μ l of competent cells, and placed on ice for 25mins.
- 5) The transformation mixture was then transferred to 37°C for 2mins and then left at room temperature for 10mins.
- 6) 1ml of L-broth was added to the mixture which was incubated at 37°C with shaking for 60-90mins.
- 7) The cells were pelleted in an Eppendorf micro-centrifuge for 20secs and resuspended in 200 μ l of L-broth.
- 8) For recombinant DNA cloning procedures, 50 μ l of this solution was spread plated onto selective medium.
- 9) For plasmid DNA transformation, 100 μ l of this solution was suitably diluted in isotonic saline, and 0.1ml was spread plated onto appropriate antibiotic selective medium.

10) Competent cells untransformed with plasmid DNA were treated in the same manner as above, and 0.1ml was spread plated onto the appropriate selective medium as a control.

2.29 Estimation of plasmid copy numbers

Plasmid copy number determinations of cells sampled from continuous culture experiments, were carried out by R.G. Chapman of G.D. Searle & Co. Ltd., High Wycombe, and involved quantification of the amount of plasmid DNA in a 1% (w/v) agarose gel by microdensitometry.

Preparation of whole cell DNA was essentially that as described by Twigg and Sherratt, (1980) and carried out as follows:

- 1) 50 μ l of lysis solution containing 10% (w/v) Ficoll (Sigma Chemical Co. Ltd., type 400), 5% (w/v) sodium dodecyl sulphate and 0.01% (w/v) bromophenol blue was added to a suspension containing about 10^9 organisms per ml and thoroughly mixed.
- 2) The mixture was incubated in a water bath at 65°C for 30mins, at the end of this time 5 μ l of ribonuclease A (1mg/ml), (Sigma Chemical Co.Ltd., in 5mM tris pH8.0), which had been incubated at 95°C for 10mins to remove DNase, was added.
- 3) This final mixture was vortexed on a 'whirlimixer' at top speed for exactly 30secs (the timing of this stage of the operation is critical).

4) To reduce the effects of 'tail-back' and smearing on the gel, this preparation was diluted 1 in 10 with lysis solution, and 50 μ l of this final solution was loaded onto a 1% (w/v) agarose gel prepared in tris-acetate buffer. Following electrophoresis, the gel was stained with ethidium bromide, and photographed under a 302nm U.V.-light source.

5) Each electrophoresis track imprinted on the photographic negative, was traced onto a sheet of paper by a microdensitometer scanner (Joyce-Loebel).

6) The copy number of a plasmid was obtained by carefully cutting from the paper trace the chromosome and plasmid peaks, and weighting them on a balance accurate to 0.1mg. The ratio in grams of the plasmid peak and the chromosomal peak, multiplied by the mass of the bacterial chromosome in kilobase pairs (assumed to be 3,800kb), was divided by the mass in kilobase pairs of the particular plasmid concerned, giving an estimate of the plasmid copy number.

2.30 Operation of the chemostat

a) Continuous culture medium

For chemostat culture the basal salts medium (Jones et al., 1980b), consisted of the following:

NH ₄ Cl	-	1g
MgSO ₄ .7H ₂ O	-	1g
CaCl ₂ (anhydrous)	-	0.1g
Fe ³⁺ citrate	-	1.8mg
Distilled H ₂ O	-	1 litre

This medium was supplemented with required amino acids and vitamins, at 60µg/ml and 20µg/ml final concentrations respectively.

Glucose (20% w/v), was sterilized separately by autoclaving and added to sterile medium to give a final concentration of 0.2g/l (glucose-limited cultures), or 5g/l (phosphate-limited cultures). Phosphate was supplied as 0.1M potassium phosphate buffer pH7.0, sterilized separately by autoclaving and added to sterile medium to give a final concentration of 5% (v/v) or 0.1% (v/v) for glucose-limited and phosphate-limited cultures respectively.

For phosphate-limited continuous culture at 42°C, the concentration of all medium constituents were increased 5-fold.

For ammonia-limited cultures the NH_4Cl concentration was reduced to 27mg/l and 1g/l of NaCl was added.

Medium was autoclaved in 20 litre quantities, and when cool, sterile phosphate and glucose were added to the necessary final concentrations.

b) Apparatus

The chemostat employed during the course of this study was the commercially available 'Bioflo' model C30 Chemostat (Fig. 2.1), (New Brunswick Scientific Co. Ltd., Edison, New Jersey, USA). The working culture vessel volume of this model is about 350ml. Medium was supplied to the culture vessel from a 20 litre reservoir at a flow-rate determined by a separate peristaltic pump (HR Flow inducer, supplied by Watson-Marlow Ltd.,

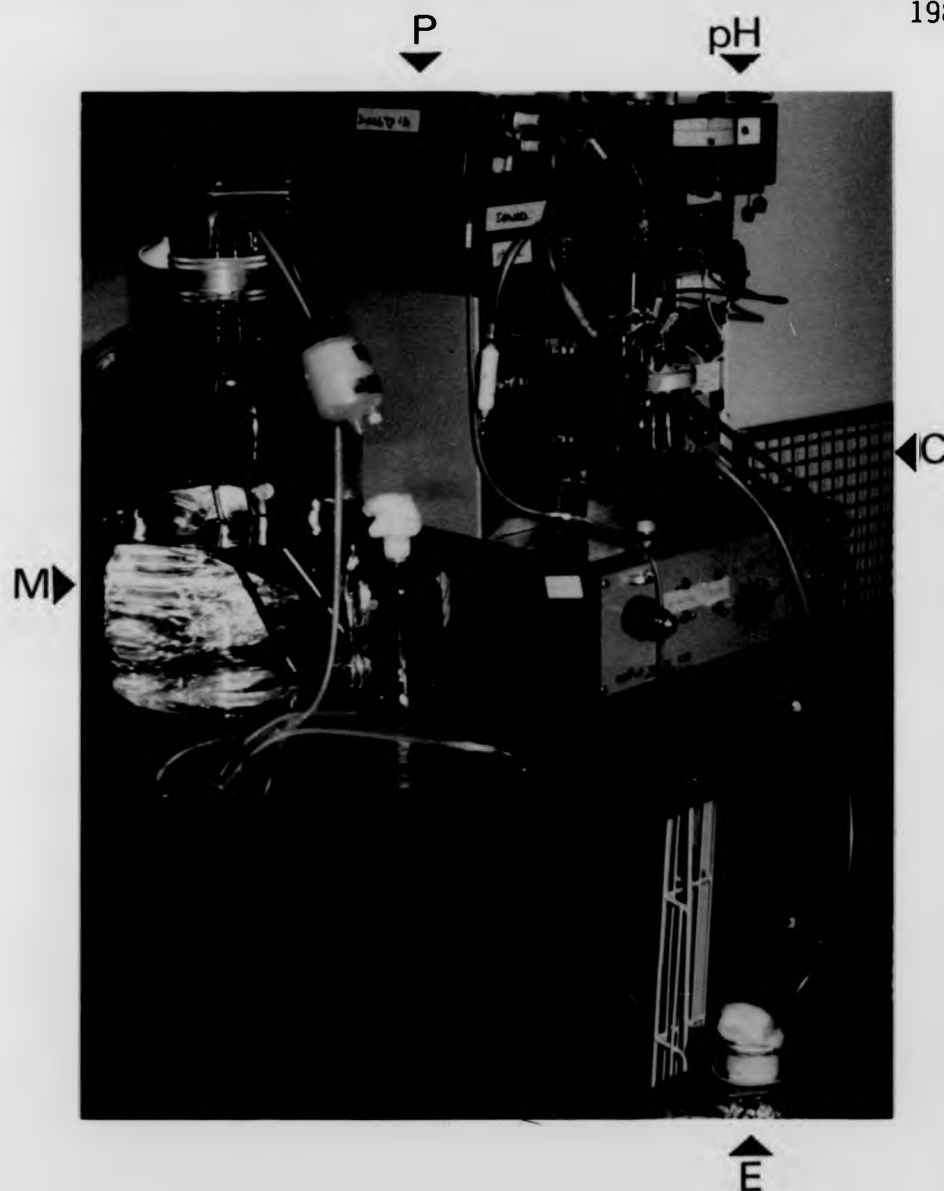


Figure 2.1:

A 'Bioflo' model C30 chemostat employed during the course of this study. The following peripheral equipment is indicated; 20 litre sterile medium reservoir (M), peristaltic pump (P), pH control apparatus (pH), culture vessel (C) and effluent reservoir (E).

Falmouth, Cornwall, England).

c) Culture conditions

Temperature was normally maintained at 37°C unless otherwise stated by an inbuilt thermostatic immersion heater. The cultures were maintained at pH7.0 by a pH probe attached to a separate pH meter (model 91B, Electronic Instruments Ltd., Chertsey, Surrey, England), which automatically regulated the pH of cultures by addition of 2M NaOH. Cultures were evenly mixed by the rotation of a bladed magnetic flea attached to the base of the culture vessel, and air sterilized by passage through a cotton wool filter, was delivered to the culture vessel by an inbuilt airpump and flow regulator.

d) Chemostat culture operation

Bacteria to be used as inocula were grown from an overnight culture in 10ml L-broth, supplemented with an appropriate antibiotic for selection of the particular plasmid under study. Following 8hrs incubation at 37°C, this culture was used to inoculate an antibiotic-free chemostat. After 16hrs of batch growth the flow-rate of medium was set at an appropriate dilution rate, normally 0.2hr^{-1} , unless otherwise stated. The flow-rate of medium was measured by timing the flow of medium from a 10ml graduated pipette, located between the medium reservoir and the peristaltic pump. This value (in ml hr^{-1}), was then used to calculate the dilution rate of the culture from the formula:

$$D = \frac{f}{v}$$

where D = dilution rate (hr^{-1})

f = flow rate (ml hr^{-1})

v = working volume of the culture vessel

At 'steady state' D is equal to μ (the specific growth rate). In order to calculate generation time, it was assumed that 'steady state' conditions were attained at the initiation of medium flow. The dilution rate was therefore used to calculate generation time from the following formula:

$$gt = \frac{\ln 2}{D} = \frac{0.693}{D}$$

where gt = generation time

e) Screening for plasmid-free segregants

Samples were removed from the chemostat at several generation intervals, via a sample port connected to the culture vessel. Cells from 10ml of the culture fluid were centrifuged at 5,000rpm (MSE Chilspin, 6 x universal-angled rotor), for 10mins and resuspended in 1ml of a sterile 0.1M potassium phosphate buffer pH7.0, containing 20% (v/v) glycerol, and stored at -70°C until required for plasmid copy number determination. Viable count determinations were performed on L-agar using 0.1ml of the culture sample, after suitable dilution in isotonic saline. About 200

colonies from each viable count determination were scored for antibiotic resistance markers, by using sterile toothpicks to transfer colonies onto L-agar and L-agar containing the appropriate antibiotic. Any presumptive plasmid-free segregants together with plasmid-bearing strains, were periodically examined for plasmid content using the rapid, small-scale plasmid isolation method described in Section 2.21.

f) Detection of culture contamination

In addition to the visual identification of coliform colonies on L-agar, colonies were also scored at various time intervals for resistance to streptomycin, which E.coli strains W5445 and WX100 carried as chromosomal markers. Contamination of continuous cultures rarely occurred.

g) Cell number and volume

In some instances both cell numbers and volumes were monitored by the use of a Coulter counter and Channelizer (Coulter Electronics Limited, Luton, Bedfordshire, England). Samples were suitably diluted in 'Isoton', as supplied by the manufacturer, and counted under conditions recommended by the vendor.

h) Phosphate determination

Detection of phosphate in the supernatants of culture samples during phosphate limitation, were carried out using the basic methodology of Deuel et al. (1970):

- 1) 0.2ml of sample supernatant was added to 1.8ml distilled H₂O.
- 2) Standard solutions of phosphate were prepared by diluting, in distilled H₂O, a 10µg/ml solution of potassium dihydrogen phosphate, each dilution should have a final volume of 2ml.
- 3) 2ml of the following reagent was added to each 2ml of sample and standard:

1 vol.	-	6M H ₂ SO ₄
1 vol.	-	2.5% ammonium molybdate
2 vol.	-	distilled H ₂ O
1 vol.	-	10% ascorbic acid (must be added last).

4) The mixtures were incubated at 37°C for 2hrs, and the optical density read at 820nm. The amount of phosphate present in the sample was determined from a plot of OD_{820nm} against standard phosphate concentrations.

i) Glucose determination

For glucose-limited cultures the detection of freely available glucose in the culture fluid was monitored by use of the commercially available 'Clinistix', a diagnostic agent for the detection of glucose in urine (Ames Division, Miles Laboratories Ltd., P.O.Box 37, Stoke Court, Stoke Poges, Slough, England).

j) Assays for other nutrients

Both spent and fresh medium were routinely analysed by the analytical section, CAMR, Porton Down, Wiltshire, for both organic and inorganic constituents.

CHAPTER III

THE SEGREGATIONAL STABILITY OF COLE1-TYPE PLASMIDS DURING CHEMOSTAT CULTURE

3.1 Introduction

Almost all attempts to achieve the efficient expression of foreign genes in E.coli have been made using plasmid vectors, the majority of which contain the replication elements of the naturally occurring plasmid pMB1, closely related to plasmid ColE1 (Baghwat & Person, 1981). Previous work has shown that in contrast to ColE1, the widely used cloning vector pBR322, exhibits structural stability but segregational instability (Jones et al., 1980b). Although maintenance of a plasmid-containing population can be ensured by inclusion of antibiotics in the growth medium, there are in some instances, regulatory and economic reasons why this cannot be undertaken on a large scale. It would therefore be advantageous to those seeking the commercialization of cloned gene products, if alternative methods could be developed for the stabilization of ColE1-type cloning vectors.

One method that could be adopted involves the use of an appropriate selectable marker, excluding antibiotic markers, the loss of which results in death of the host cell. Such a strategy may make use of auxotrophic complementation. However, since naturally occurring plasmids are themselves stably maintained, an alternative strategy could be to employ naturally occurring plasmid stability functions, thus ensuring stable inheritance. The former, in contrast to the latter strategy, relies on plasmid retention within a population through the death of plasmid-free cells and may therefore incorporate some degree of inefficiency.

This chapter describes studies carried out to investigate the stabilization of plasmid pBR322, by approaches similar to those outlined above. The segregational instability of pBR322 during chemostat culture was employed as an assay to examine various cloned sequences, and determine their potential for conferring segregational stability. However, it became apparent that the manipulation of plasmid DNA sequences can have a direct bearing on the segregational stability of a recombinant plasmid, unrelated to functions associated with the cloned DNA.

3.2 Partial stabilization of plasmid pBR322 by auxotrophic complementation

3.2.1 Introduction

Ammonia assimilation by E.coli K-12 involves two pathways, A) glutamine synthetase (GS)/glutamate synthase (GOGAT), and B) glutamate dehydrogenase (GDH), (Fig.3.1). Strains lacking GDH do not require glutamate for growth, and such mutants are able to grow as well as wild type strains on both low and high concentrations of ammonia. On the other hand, strains lacking GOGAT can grow without glutamate, but are unable to grow on low concentrations of ammonia. In the absence of GOGAT, glutamate is supplied to glutamine synthetase by GDH. In wild-type strains, when the supply of ammonia is limited, the GDH pathway is unable to function efficiently and ammonia is assimilated via the GS/GOGAT pathway. Mutants that lack both GDH and GOGAT cannot of

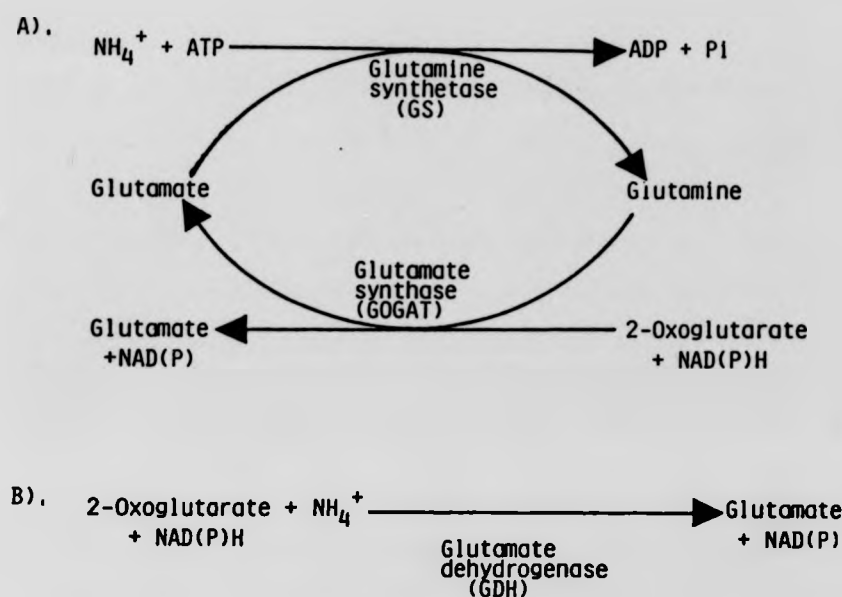


Figure 3.1

Pathways of ammonia assimilation in E.coli K-12. A)

GS/GOGAT, and B) GDH. The K_m for ammonia of the enzymes GS and GDH are 1mM and 10mM respectively.

(Reproduced from Windass et al., 1980).

course utilize ammonia, and therefore require glutamate for growth (Tyler, 1978).

3.2.2 Results

Windass *et al.* (1980) using auxotrophic complementation, have succeeded in cloning a region of the *E.coli* chromosome encoding GDH. *E.coli* K-12 strain JW114, a plasmid bearing derivative of strain CB100, is deficient in both GDH (*gdh*) and GOGAT (*gltB*), and normally requires glutamate for growth, however, by virtue of the resident pACYC184/*gdh*⁺ plasmid, JW114 is glutamate-independent. Since this recombinant plasmid had been positively selected for by auxotrophic complementation, and as the K_m of GDH for ammonia is relatively high, it was envisaged that re-cloning and expression of the GDH enzyme in pBR322, would lead to segregational stability and increased copy number when host cells with an appropriate selective background (such as strain CB100), were cultured under ammonia-limiting chemostat conditions. Initially, therefore, plasmid DNA was isolated from strain JW114 by CsCl-EtBr density gradient centrifugation.

This preparation of plasmid pACYC184/*gdh*⁺ as expected, generated two restriction fragments when digested with *Sal*I (Fig. 3.2), a 4.26kb fragment specifying chloramphenicol resistance and pACYC184 replication control, and a 7.6kb fragment encoding GDH. This latter fragment was re-cloned into pBR322, by using *Sal*I restricted preparations of pACYC184/*gdh*⁺ and pBR322 DNA, which had been mixed and ligated, and transformed

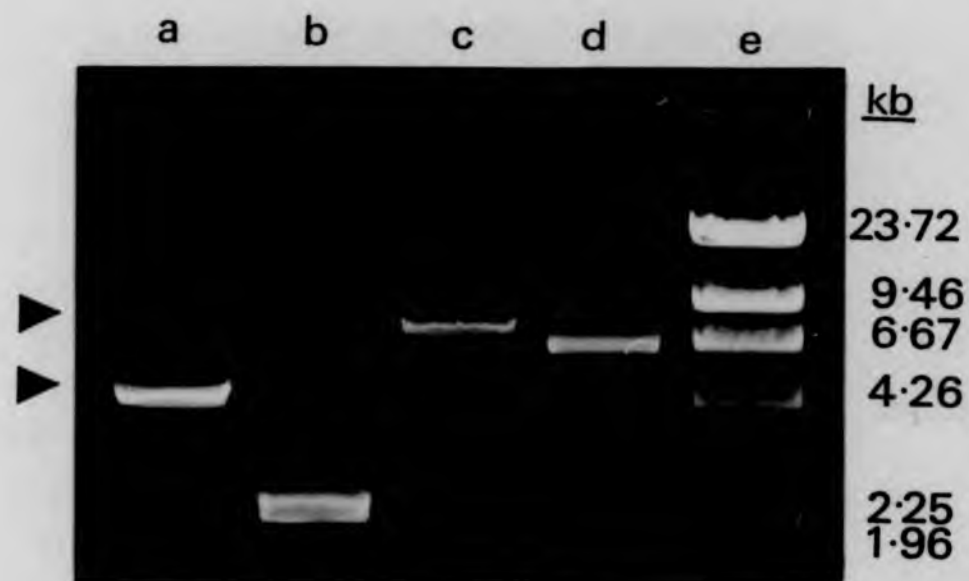


Figure 3.2

Comparative *SalI* restriction endonuclease analysis of plasmids pBR322 and pACYC184/*gdh*⁺. Lanes a and b) *SalI* digested and undigested pBR322 DNA, c and d) *SalI* digested and undigested pACYC184/*gdh*⁺ DNA, e) *Hind*III digest of lambda DNA.

into strain CB100, selection was made for ampicillin resistance and glutamate independence (Fig.3.3 A & B).

Rapid, small scale plasmid analysis of fourteen ampicillin-resistant, glutamate-independent, tetracycline- and chloramphenicol-sensitive transformants of CB100 (Fig.3.3 C), indicated that except for a single clone, all plasmids co-migrated on agarose gels with pACYC184/gdh⁺, as would be expected from the predicted combined size of a pBR322/gdh⁺ recombinant plasmid. Plasmid DNA from six of the ampicillin-resistant, glutamate-independent transformants, carrying an appropriately sized pBR322 recombinant plasmid, were isolated and purified by CsCl-EtBr density gradient centrifugation, following chloramphenicol amplification, and their size confirmed using agarose gel electrophoresis (Fig.3.4). One of the recombinant plasmids was chosen for further analysis and designated pWX15. Transformation of strain WX100 with pWX15 conferred glutamate independence and ampicillin resistance.

Strain WX100 is a recA56 derivative of CB100, which was constructed by Pl-mediated transduction of the recA56 locus linked to transposon Tn10. Tetracycline-resistant transductants were screened for nitrofurantoin and U.V. sensitivity, as well as glutamate dependence. Rapid, small scale plasmid isolation and analysis in agarose gels, however, indicated that pWX15 exhibits an altered migrational pattern when isolated from strain WX100 (Fig.3.5).

The effects of ammonia limitation on the stability of pWX15 in strain WX100 was investigated. Plasmid

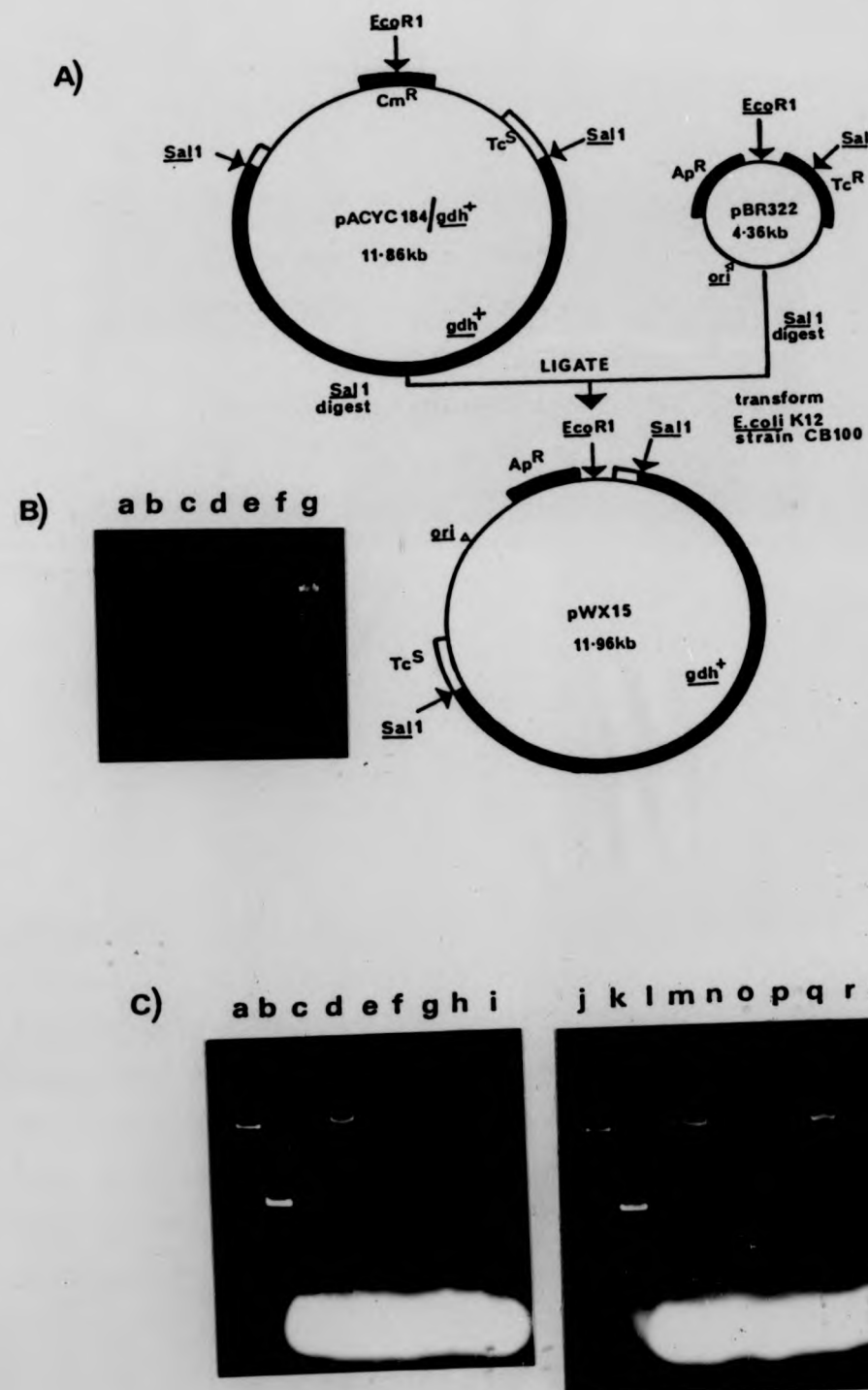
Figure 3.3

Re-cloning of a 7.6kb fragment of *E.coli* K-12 genomic DNA encoding glutamate dehydrogenase (*gdh*), from pACYC184/*gdh*⁺ to pBR322, generating pWX15.

A) Illustration of the re-cloning protocol.

B) Agarose gel electrophoresis of treated plasmid DNA preparations during the re-cloning procedure, lane a) *Sal*I digested pBR322 DNA incubated with T4 DNA ligase, b) *Sal*I digested pBR322 DNA unligated, c) *Sal*I digested pBR322 and pACYC184/*gdh*⁺ DNA, mixed and ligated with T4 DNA ligase, d) *Sal*I digested pBR322 and pACYC184/*gdh*⁺ DNA, mixed and unligated, e) *Sal*I digested pACYC184/*gdh*⁺ DNA incubated with T4 DNA ligase, f) *Sal*I digested pACYC184/*gdh*⁺ DNA unligated, g) *Hind*III digest of lambda DNA.

C) Agarose gel electrophoresis of rapid, small-scale plasmid preparations obtained from fourteen ampicillin-resistant, glutamate-independent, tetracycline- and chloramphenicol-sensitive transformants of strain CB100, lanes a and j) pACYC184/*gdh*⁺ DNA, b and k) pBR322 DNA, c to h, and l to s) rapid plasmid preparations of strain CB100 transformants, i) pAT153 DNA.



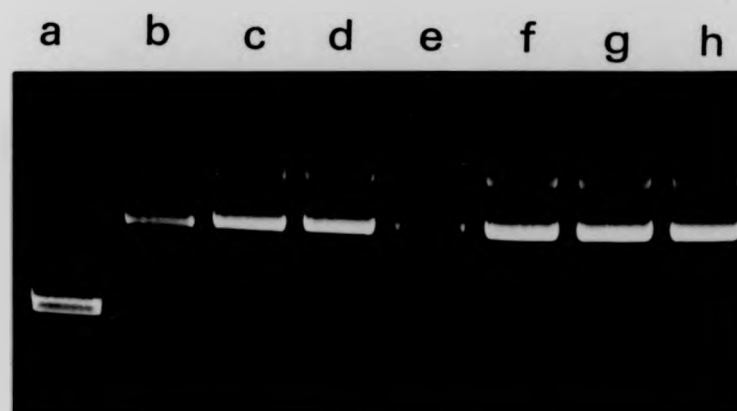


Figure 3.4

Agarose gel electrophoresis of purified plasmid DNA preparations obtained from six ampicillin-resistant, glutamate-independent transformants of strain CB100.

Lane a) pBR322 DNA, b) pACYC184/gdh⁺ DNA, and lanes c to h) plasmid DNA preparations of strain CB100 transformants harvested from CsCl-EtBr density gradients.



Figure 3.5

Agarose gel electrophoresis of plasmid preparations obtained from ampicillin-resistant and sensitive strains of WX100, isolated during ammonia-limited chemostat culture of strain WX100 bearing plasmid pWX15. Lanes a and g) purified plasmid DNA from strain CB100 bearing plasmid pWX15, isolated following CsCl-EtBr density gradient centrifugation, b) rapid, small-scale plasmid preparation from strain WX100 bearing plasmid pWX15, c and d) rapid, small-scale plasmid preparations, from respectively, ampicillin-sensitive and resistant strains of WX100 isolated following 78 generations of ammonia-limited chemostat culture, e and f) rapid, small-scale plasmid preparations, from respectively, ampicillin-resistant and sensitive strains of WX100 isolated following 2 generations of phosphate-limited chemostat culture.

pWX15-free cells were found to segregate rapidly during nonselective phosphate-limited chemostat culture (Fig. 3.6). At time₀, when medium flow was initiated, only about 26% of the culture population still retained pWX15. After 30 generations of chemostat culture, no plasmid-bearing cells could be detected. In contrast, ammonia-limited chemostat culture resulted in the persistence of pWX15 for 190 generations. However, from 20 generations onwards, a steady decline in the proportion of plasmid-bearing cells was observed (Fig. 3.6). Rapid, small scale plasmid analysis of both ampicillin-resistant and sensitive colonies (Fig.3.5), revealed that the loss of ampicillin resistance appeared to be due to the segregation of plasmid-free cells. Furthermore, cells that were plated directly from the ammonia-limited chemostat onto rich medium, exhibited differences in their colony morphologies (Fig.3.7). Two distinguishable colony types were observed, either a small, circular, well defined colony having a convex elevation, or a large, circular, raised colony possessing a characteristic 'pie crust' outline. In contrast, cells obtained from phosphate-limited chemostat culture, when plated onto rich medium, did not exhibit such marked morphological colony differences. The different colony types arising from ammonia-limited chemostat culture at about 100 generations, were tested for their sensitivity to ampicillin. The majority of large colonies proved to be ampicillin-sensitive, whilst most small colonies were ampicillin-resistant. All colonies tested were resistant to streptomycin,

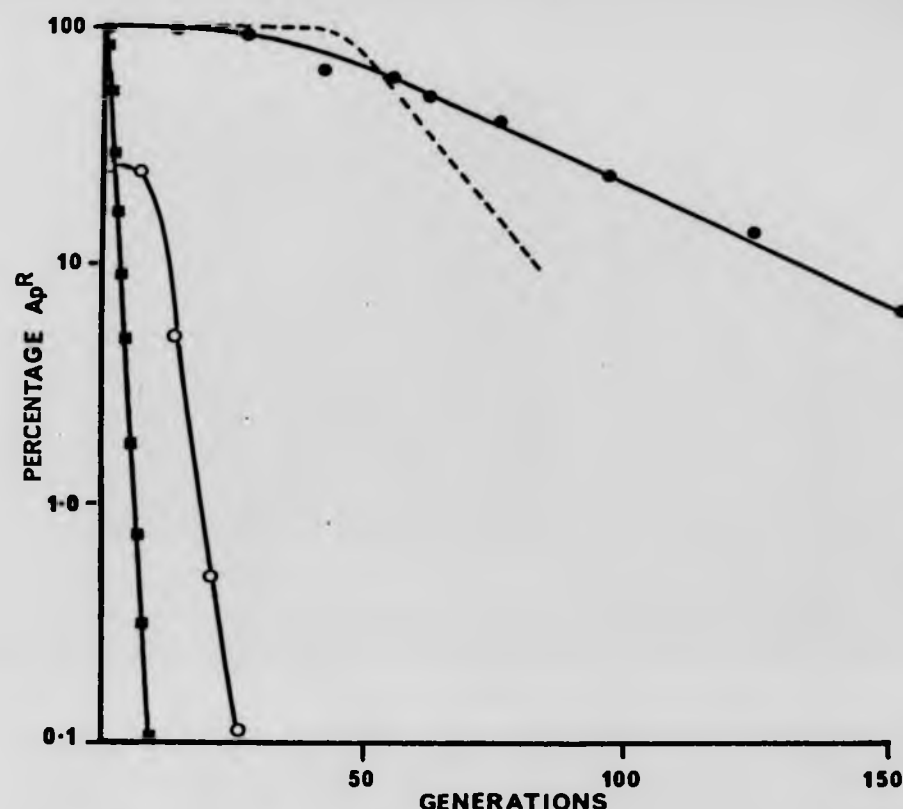


Figure 3.6

Persistence of plasmid pWX15 in *E. coli* strain WX100.

The results are presented as a semi-logarithmic plot of percentage ampicillin-resistant population with number of generations, during either; ●—●, ammonia

or; ○—○, phosphate-limited chemostat culture.

Data showing the persistence of plasmid pBR322 in *E. coli* strain W5445 during phosphate-limited chemostat culture; -----, is taken from Jones *et al.* (1980b).

In all three experiments the dilution rate was 0.2hr^{-1} (a mean generation time of about 3.47hrs.); ■—■,

theoretical percentage washout of plasmid-bearing organisms at $D = 0.2\text{hr}^{-1}$.

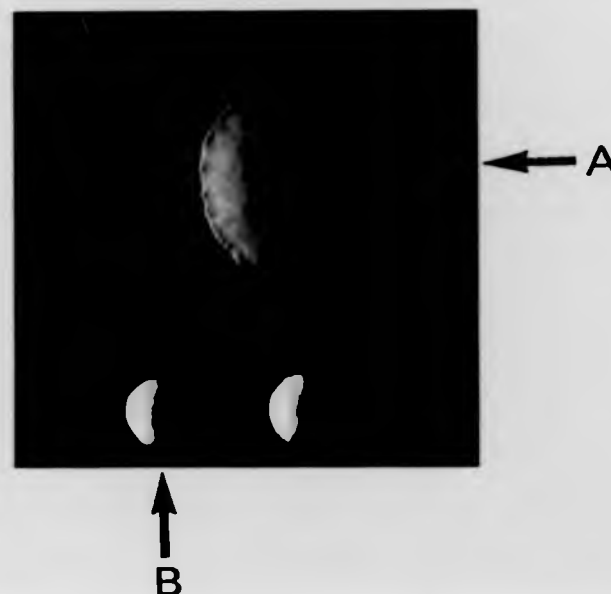


Figure 3.7

Colony morphologies of E.coli strain WX100 cells, plated directly onto rich medium following about 153 generations of ammonia-limited chemostat culture.

A) Characteristic ampicillin-sensitive, large circular 'pie crust' colony.

B) Small circular ampicillin-resistant colony.

(Magnification, 10-fold).

excluding the possibility of culture contamination.

Twenty plasmid-free colonies arising from each of the phosphate- and ammonia-limited chemostat cultures, at about 80 and 160 generations respectively, were assayed for glutamate-dependence. All twenty plasmid-free colonies arising in the ammonia-limited chemostat were glutamate independent, and likewise, sixteen of the twenty plasmid-free colonies arising in the phosphate-limited chemostat were also glutamate-independent. Cells from all forty colonies were ampicillin-sensitive and plasmid-free, as determined by rapid, small scale plasmid isolation and visualization in agarose gels.

3.2.3 Concluding remarks

In cloning the GDH gene of E.coli K-12, the ultimate aim of Windass et al. (1980) was the efficient production of single cell protein (Pruteen), during fermentation of the bacterium Methylophilus methylo-
trophus. It was reasoned that since assimilation of ammonia via GDH is more energy-efficient than via GOGAT (i.e., requires one less molecule of ATP per molecule of glutamate formed), and that M.methylo-
trophus does not possess a chromosomal gene for GDH, then growth on ammonia of a strain carrying a suitable mutation in the gene for GOGAT, should lead to the retention of a plasmid specifying the GDH gene of E.coli and consequently a more efficient fermentation. Indeed, during continuous culture of a 250,000 litre fermenter operating at a dilution rate of 0.2hr⁻¹ for over six

months, no plasmid-free segregants of such a strain were detected (Powell & Byrom, 1982). However, caution should be exercised when interpreting results relating to plasmid maintenance during ammonia limitation. Other workers have reported that pBR325-bearing cells persist during ammonia-limited chemostat cultures, in contrast to other nutrient limitations (Noak *et al.*, 1981), and that R6-bearing cells possess an unknown selective advantage over isogenic plasmid-free cells during ammonia limitation (Wouters & van Andel, 1983). These findings would suggest that competition between plasmid-free and plasmid-bearing cells is less stringent during ammonia than other nutrient limitations, and given a selective advantage, plasmid-bearing cells should predominate.

It is therefore notable, that during phosphate limitation, no pWX15-bearing cells could be detected following 30 generations of continuous culture. This is in contrast to similar chemostat cultures of segregationally unstable plasmids, such as pBR322 (Jones *et al.*, 1980b), where a residual population (<1%), of plasmid-bearing cells were always maintained. It would appear, therefore, that during phosphate- in contrast to ammonia-limited chemostat culture, positive selection occurred against cells of strain WX100 bearing pWX15.

However, it is also evident that on subsequent transformation into strain WX100, plasmid pWX15 exhibits an altered migrational pattern in agarose gels (Fig. 3.5). Since strain WX100 is recombination deficient,

and no obvious ladder of multimers was observable following gel electrophoresis, then one possible explanation accounting for an increase in the size of pWX15 may be as a consequence of recA-independent transposition of Tn10, present within the host background of strain WX100. Bearing in mind the increased size of pWX15 and the possibility of DNA rearrangement as a consequence of the presence of Tn10, then the marked persistence of this 'pWX15 derivative' during ammonia-limited chemostat culture, in contrast to nonselective phosphate-limited chemostat culture, may suggest that a similar degree of plasmid maintenance, to that reported for M.methylotrophus, might also be attainable employing E.coli K-12 as a host cell.

However, the reversion of strain WX100 to glutamate independence, which occurred during phosphate as well as ammonia limitation, poses an additional problem to the segregational stability of pWX15. Indeed, E.coli strain CB100 (PA340), the parent strain of WX100, has been reported to revert to glutamate independence at a frequency of 10^{-8} per cell (Covarrubias et al., 1980). Modification of the host background by introduction of chromosomal deletions in both the gdh and gltB genes, should reduce the frequency with which this strain reverts to glutamate independence. However, a reversion to glutamate independence by integration of pWX15 into the genome of WX100, and rapid plasmid loss during phosphate limitation, may have occurred as a consequence of recA-independent recombination, mediated by either

the postulated Tn10 insertion, or a recombinogenic locus located within the gdh fragment. It has recently been shown that two repetitive extragenic palindromic (REP) sequences are located at the 3'-end of the gdhA gene of E.coli K-12 (Becerril et al., 1985). This gene encodes the 447 amino acid polypeptide monomer of the hexameric GDH enzyme (McPherson & Wootton, 1983). REP sequences represent a family of dispersed sequences occurring within the genome of E.coli (Gilson et al., 1984). Several functions have been suggested for these sequences in the control of gene expression at the translational level (Stern et al., 1984). However, in certain instances it may be possible that such palindromic sequences may also serve as sites for recombination (Warren & Green, 1985). In this context it should be noted that phosphate limitation may lead to an induction of the host-integration factor himA (Wanner, 1983), which is required for integration and excision of phage lambda. Therefore, differences in nutrient limitation may lead to differences in the expression of recombination pathways. Unfortunately, glutamate-independent plasmid-free colonies were not tested for low levels of ampicillin resistance, and therefore the contribution that pWX15 integration may have made towards reversion to glutamate independence, and plasmid loss, could not be determined.

Deletion of plasmid-encoded REP sequences may prevent or reduce any postulated plasmid integration into host backgrounds similar to strain WX100. Deletion of one such REP sequence at the 3'-end of the gdhA gene,

has been shown to result in a decrease in upstream gene expression (Becerril et al., 1985). Since GDH has a low affinity for its substrate, there was the possibility that during ammonia limitation an increase in the copy number of pWX15 may have occurred, increasing gdh gene dosage and therefore expression. Unfortunately, plasmid instability ruled out plasmid copy number analysis.

Finally, the difference in colonial morphology of cells of strain WX100 when plated onto rich medium, following chemostat culture, appeared to correlate with the segregation of plasmid-free cells. These colony differences may reflect some physiological difference occurring between plasmid-bearing and plasmid-free cells, as a consequence of plasmid loss. However, similar colony morphology differences were also observed during subsequent chemostat cultures investigating the segregational stability of other pBR322 derivatives. A more detailed discussion of the phenotypic characteristics of these strains is presented in Chapter IV.

3.3 Segregational stability of pBR322 derivatives encoding par regions from plasmids pSC101 and R1

3.3.1 Introduction

The employment of selective pressures may represent an inefficient, and limited approach to the ensured segregational stability of plasmid cloning vectors. Naturally occurring plasmid functions preventing segregational instability, may in contrast, constitute a

more efficient and flexible approach to the maintenance of a plasmid-containing population.

3.3.2 Results

a) par region of plasmid pSC101

Meacock & Cohen (1980) have identified and characterized a genetic function designated par, which ensures the fidelity of segregation of plasmid pSC101 to daughter cells. This function has been localized within pSC101, to a 270bp fragment adjacent to the replication origin. The par locus, which appears to be functionally equivalent to the centromere of eukaryotic cells, was able to rescue unstable pSC101-derived replicons, and an unrelated par⁻ multicopy replicon, pACYC184, in the cis but not the trans configuration. The par locus in pSC101 functions independently of copy number control, and does not appear to be associated directly with plasmid replication functions.

The par region of pSC101 was re-cloned by I.M. Jones of this laboratory, into plasmid pBR322, generating plasmid pWX9 (Fig.3.8). (The cloning procedure involved an EcoRI/AvaI restriction digest of plasmid pPM31 (a p15A derivative carrying the EcoRI/AvaI par⁺ fragment from pSC101), and pBR322 (Fig.3.9). DNA was mixed, ligated and transformed into E.coli strain W5445. Tetracycline-sensitive clones were screened to determine whether the 375bp pPM31 derived EcoRI/AvaI par⁺ region, had substituted for the 1.42kb EcoRI/AvaI tetracycline-resistant coding region of pBR322).

Plasmid pWX9 was found to be stably maintained

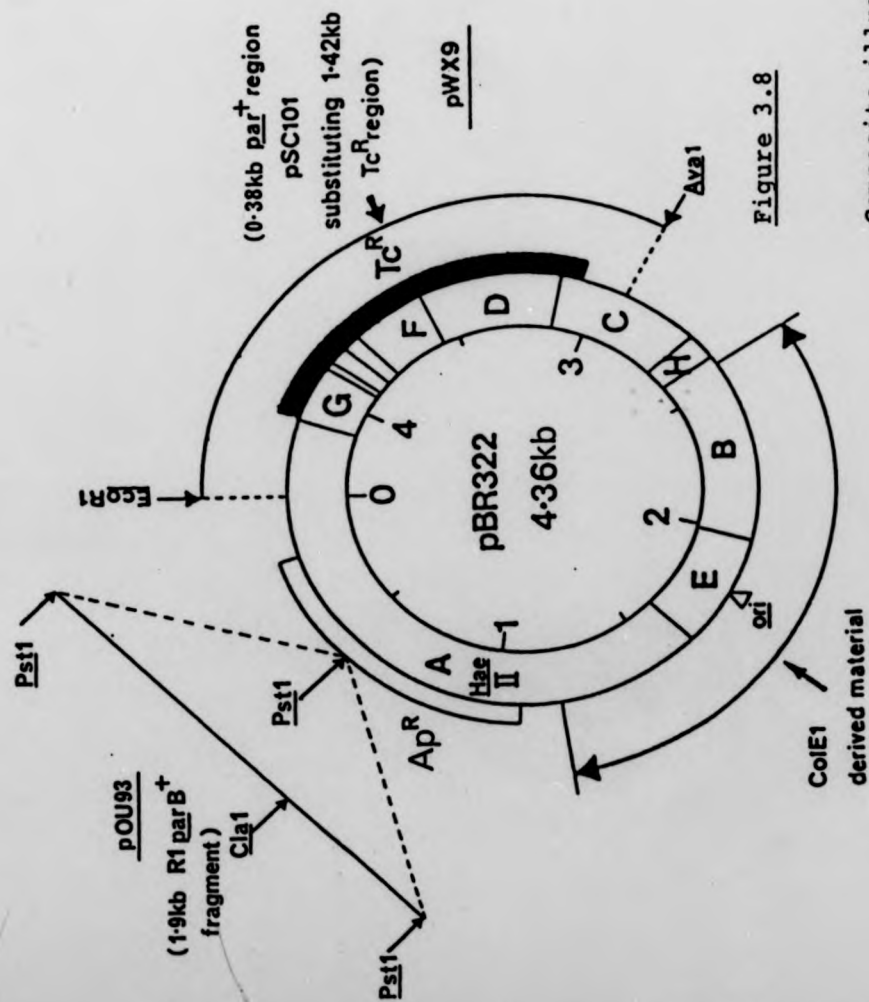


Figure 3.8

Composite illustration of recombinant pBR322 plasmids

encoding stability functions derived from either, plasmids pSC101 (pWX9), or R1 (pOU93).

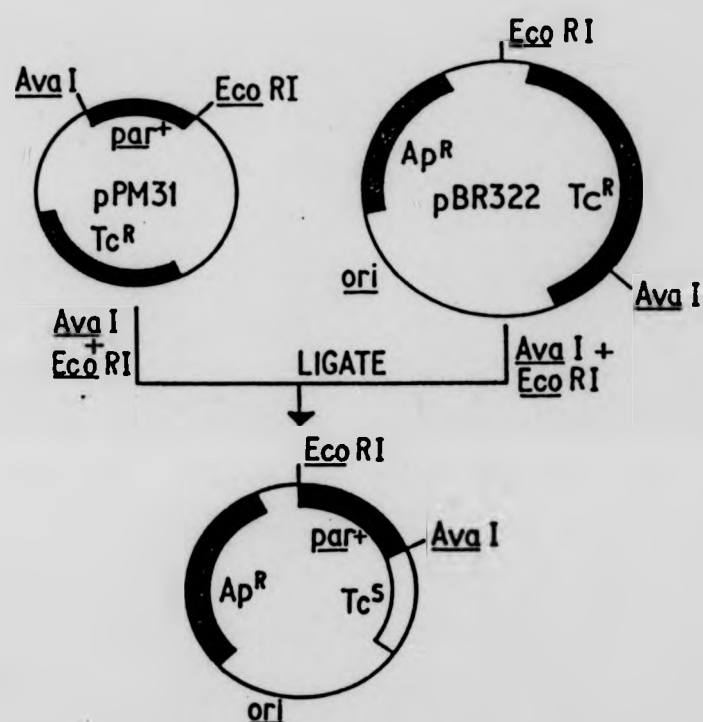


Figure 3.9

Re-cloning of the pSC101 **par⁺** fragment from pPM31 to pBR322 generating plasmid pWX9. (Reproduced from Primrose *et al.*, 1984).

during phosphate-limited chemostat culture at a temperature of 37°C and a dilution rate of either 0.05hr⁻¹, or 0.2hr⁻¹, for at least 94 and 194 generations respectively (Fig.3.10 A). In addition, pWX9 was also found to be stably maintained at a temperature of 42°C and a dilution rate of 0.2hr⁻¹ for at least 175 generations.

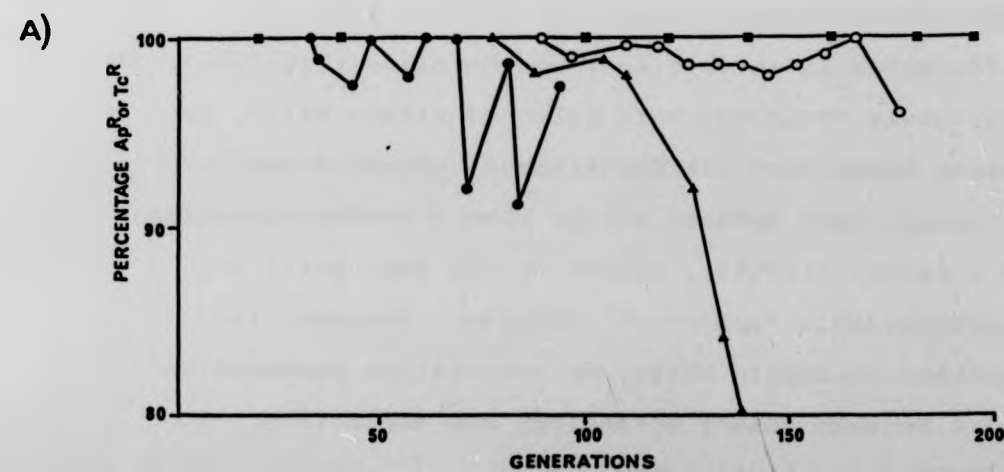
Plasmid-free segregants which did arise at about 34 generations, within the chemostat culture operated at a dilution rate of 0.05hr⁻¹, and at about 96 generations, within the 42°C chemostat culture, failed to predominate in either culture, persisting as an approximately 2% or 4% subpopulation respectively (Fig.3.10 A). The pattern of migration in agarose gels, of pWX9 DNA (Fig.3.10 B,a), obtained from whole-cell lysates of samples harvested during chemostat culture at 37°C and a dilution rate of 0.2hr⁻¹, suggests that the experimental conditions employed did not readily lead to any major structural deletion, or insertion rearrangements of pWX9 sequences.

Finally, cells from each of the chemostat cultures when plated directly onto rich medium, exhibited differences in their colony morphologies (Fig.3.11). As previously observed, with cells of strain WX100, two colony types were distinguishable, either a small, circular, well defined colony have a convex elevation, or a large, circular, raised colony possessing a characteristic 'pie-crust' outline. However, in contrast to strain WX100, no correlation appeared to exist between colony morphology and ampicillin resistance or sensitivity.

Figure 3.10

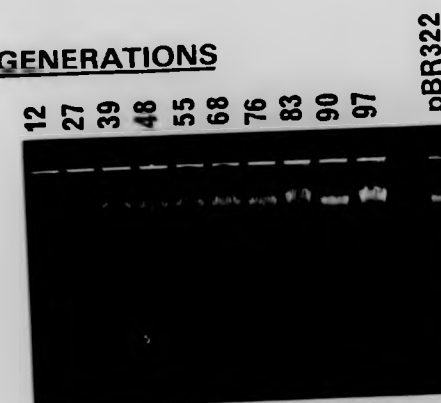
A) Persistence of plasmids pWX9 and pOU93 in *E.coli* strain W5445 during phosphate-limited chemostat culture. Results are presented as the percentage ampicillin- or tetracycline-resistant population with number of generations; ■—■, pWX9 cultured at 37°C and a dilution rate of 0.2hr^{-1} (a mean generation time of about 3.47hrs); ●—●, pWX9 cultured at 37°C and a dilution rate of 0.05hr^{-1} (a mean generation time of about 13.86hrs.); ○—○, pWX9 cultured at 42°C and a dilution rate of 0.2hr^{-1} ; ▲—▲, pOU93 cultured at 37°C and a dilution rate of 0.2hr^{-1} .

B) Agarose gel electrophoresis of whole-cell lysates, obtained from samples directly harvested during chemostat culture of pWX9- and pOU93-bearing cells (See Fig.3.13). These were subsequently used for the determination of plasmid copy number, a) pWX9 cultured at 37°C and a dilution rate of 0.2hr^{-1} , b) pOU93.

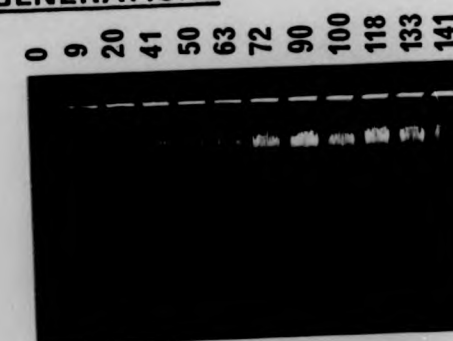


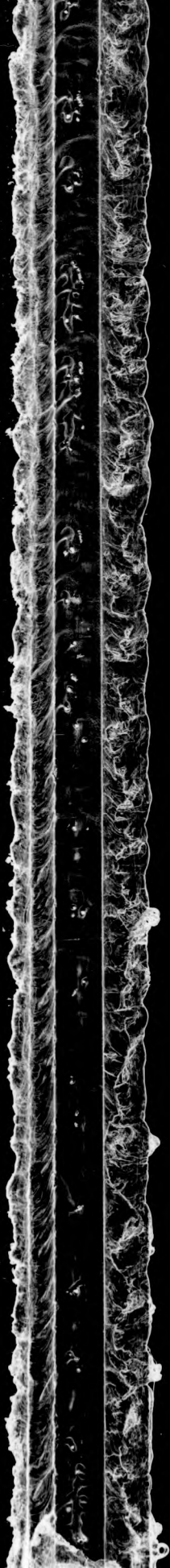
B)

a) GENERATIONS



b) GENERATIONS





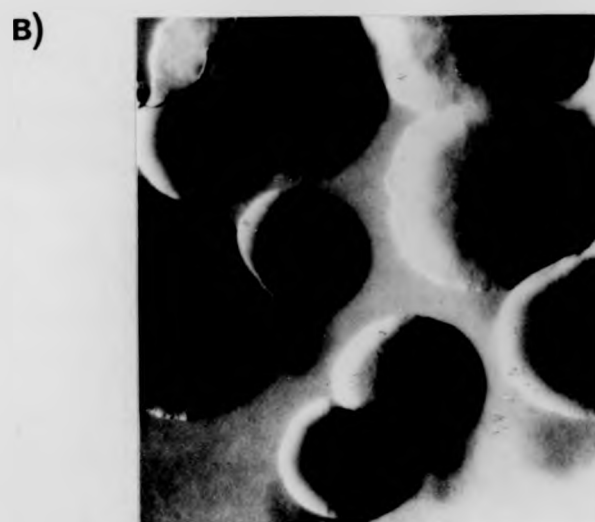


Figure 3.11

Large and small colony morphologies of E.coli strain W5445, when plated directly onto rich medium during phosphate-limited chemostat culture of pWX9-bearing cells.

A) following about 70 generations of continuous culture at a temperature of 37°C and a dilution rate of 0.05hr⁻¹,

B) following about 62 generations of continuous culture at a temperature of 42°C and a dilution rate of 0.2hr⁻¹.

(Magnification 10-fold).

b) parB region of plasmid R1

Nordstrom et al. (1980a) have suggested that the EcoRIA fragment of the large, conjugative plasmid R1, encodes a par function. Indeed, recent studies have shown that the EcoRIA fragment encodes two independent stability functions parA and parB (Gerdes et al., 1985a). The parB function appears to stabilize unrelated plasmids more effectively than the parA function, suggesting that parA may additionally require some other function associated with plasmid R1 replication. More recent evidence has suggested that the parB region may be able to couple plasmid replication to the host cell cycle (Gerdes et al., 1985b).

Plasmid pOU93 (Fig.3.8), (kindly supplied by K. Gerdes, Odense University, Denmark), is a pBR322 derivative containing a 1.9kb PstI insert, encoding the parB⁺ sequence of plasmid R1 (Fig.3.12). When assayed, pOU93 was found to be stably maintained during phosphate-limited chemostat culture for at least 90 generations (Fig.3.10 A). At about 90 generations, plasmid-free segregants arose. However, such segregants did not arise in an analogous manner to those observed for pWX9, when continuously cultured at 42°C or at a dilution rate of 0.05hr⁻¹, although differences in the colony morphology of cells plated directly from the chemostat onto rich medium were observable. Finally, pOU93 did not appear to undergo any major structural deletion or insertion rearrangements during chemostat culture, as judged by migration pattern in agarose gels



Figure 3.12

Comparative restriction endonuclease analysis of plasmid pOU93. Lane a) HindIII digest of lambda DNA, b) undigested pBR322 DNA, c) undigested pOU93 DNA, d) PstI digested pBR322 DNA, lanes e to m) pOU93 DNA incubated with either restriction endonuclease HpaI, HindIII, ClaI, BamHI, SalI, SacI, AvaI, PvuI or PstI respectively, lane n) EcoRI digested lambda DNA.

The 1.9kb R1 parB⁺ fragment of pOU93 does not appear to contain any internal sites for the restriction enzymes HpaI, HindIII, BamHI, SalI, SacI or AvaI, but does contain a single ClaI site (incubation of plasmid pOU93 with the restriction endonuclease PvuI (lane l), failed to digest pOU93 DNA, this was more than likely due to buffer conditions, since insufficient KCl was present in the incubation mix).

(Fig.3.10 B,b).

c) The copy number of plasmids pWX9 and pOU93 during chemostat culture

Plasmid copy number of chemostat cultured cells carrying either pWX9 or pOU93, was determined by analysis of the plasmid DNA content in agarose gels, of whole-cell lysates of plasmid-carrying cells harvested directly during chemostat culture and stored at -70°C (Fig.3.13). Plasmid pOU93 maintained a copy number of about 100 for at least 90 generations, at which time plasmid-free cells arose. From this period onwards, the overall reduction in copy number appears to reflect an increase in the proportion of plasmid-free cells in the sample lysate, rather than a reduction in plasmid-copy number.

In marked contrast to pOU93, plasmid pWX9 substantially increased in copy number over an initial 130 generation period of chemostat culture, from about 100 to 290. This high plasmid-copy number persisted for about a further 60 generations, at which time the chemostat experiment was ended.

3.3.3 Concluding remarks

It would appear, therefore, that the par regions from two plasmids unrelated to pBR322, R1 and pSC101, can prevent the segregational instability which pBR322 exhibits during phosphate-limited chemostat culture. However, several complicating factors do not allow the observed stability to be attributed solely to the

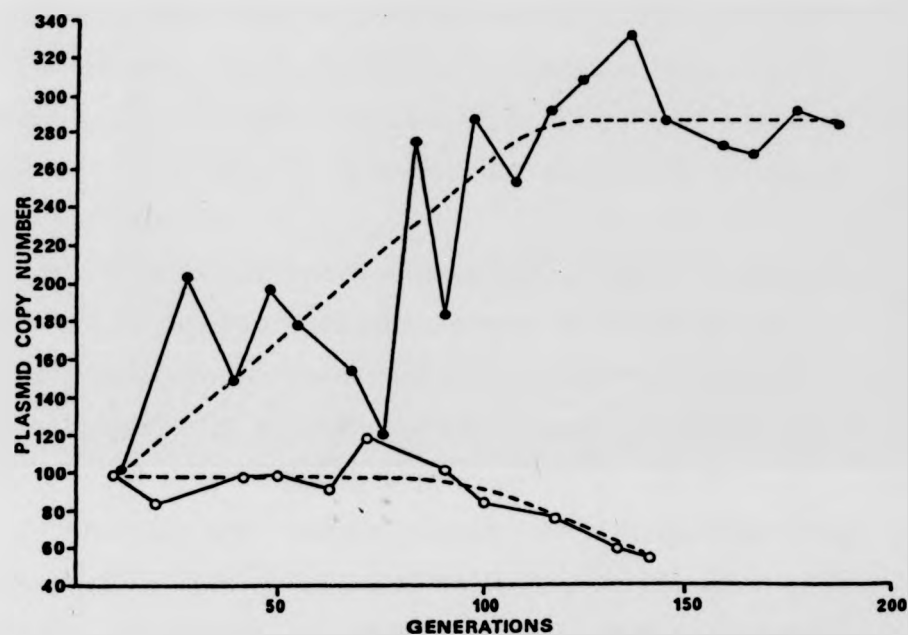


Figure 3.13

Changes in the copy number of plasmids pWX9 and pOU93 when plasmid-containing cells of *E.coli* strain W5445 were grown in phosphate-limited chemostats. ●—●, pWX9 and ; ○—○, pOU93, the overall trend in copy number variation is indicated by;-----.

respective partitioning functions. The persistence of a plasmid during nonselective chemostat culture inevitably relies on two important parameters, a) the stability of the plasmid, which may be manifested as either changes in the structure of the plasmid, or defects in plasmid maintenance, and b) the relative reproductive fitness of plasmid-bearing cells in relation to otherwise isogenic plasmid-free cells.

Plasmids pWX9 and pOU93 represent markedly different derivatives of pBR322. Plasmid pOU93 was generated following insertional inactivation of the ampicillin resistance gene, by a 1.9kb PstI fragment. Alteration of transcriptional products within pBR322, has been shown to lead to copy number changes as a consequence of increased or decreased transcriptional interference with replication functions (Stueber & Bujard, 1982). Indeed, copy number determination of pOU93 during chemostat culture, demonstrated a several-fold higher copy number than that of pBR322 (Fig.3.13), the copy number of which is estimated to be about 30 in exponentially growing cells of E.coli strain HB101 (M. Nugent, personal communication, G.D. Searle Co.Ltd., High Wycombe, 1982). In addition, pOU93 expresses tetracycline resistance, which has recently been shown to reduce the growth-rate of plasmid-bearing cells during glucose-limited chemostat culture (Lee & Edlin, 1985). Therefore, following the elapse of an appropriate number of generations, presumably dependent on the prevailing copy number, plasmid-free segregants which arise may be expected to be at a selective advantage and therefore

predominate within the chemostat population.

However, the kinetics of appearance of pOU93-free segregants were not analogous to those of pBR322-free segregants under similar chemostat conditions (Fig.3.10 A), (Jones et al., 1980b). Furthermore, a reduction in copy number due to nutrient limitation, as postulated for plasmid pBR322, did not occur (Fig.3.13), (Jones et al., 1980b). The copy number of pOU93 remained more or less constant during chemostat culture. One possible explanation may be that host mutants arose, which were defective in plasmid segregation, that were unable to compete effectively with the disadvantaged pOU93-bearing parent strain. It cannot, therefore, be categorically stated that the parB function of plasmid R1 is able to stably maintain the vector pBR322 with any greater efficiency, than that associated with an increase in plasmid copy number.

Plasmid pWX9 contrasts markedly with pOU93. Construction of pWX9 involved a substitution of the tetracycline resistance coding region, with a 375bp fragment specifying the par function of pSC101. Therefore, this pBR322 derivative may be expected to possess a copy number several-fold greater than that of pBR322 (Stueber & Bujard, 1982), which was found to be the case during chemostat culture of strain W5445 carrying pWX9 (Fig.3.13). Deletion of the tetracycline resistance region, would also have removed any growth-rate disadvantage conferred on the host cell by expression of tetracycline resistance (Lee & Edlin, 1985).

Following 194 generations of continuous culture at a temperature of 37°C and a dilution rate of 0.2hr⁻¹, no plasmid-free segregants of pWX9-bearing cells could be detected (Fig.3.10 A). Alteration of the dilution rate to 0.05hr⁻¹, or an increase in temperature to 42°C, in an attempt to vary conditions which have previously been found to be decisive in the outcome of competition experiments, between plasmid-free and plasmid-bearing cells (Wouters *et al.*, 1980), did lead to the detection of pWX9-free segregants (Fig.3.10 A). Changes in such continuous culture parameters as dilution rate or temperature would be expected to lead to alterations in specific growth-rate, and as demonstrated recently (Lin-Chao & Bremer, 1986), a decrease in specific growth-rate should lead to an increase in the copy number of ColE1-type plasmids, presumably affecting the reproductive fitness of plasmid-bearing cells in relation to otherwise isogenic plasmid-free cells. Plasmid-free segregants which did arise, were however, not isogenic with the host strain W5445, and existed as a subpopulation of variants unable to predominate within the chemostat population. These plasmid-free cells represent mutants of the host strain, with no growth-rate advantage over their plasmid-bearing parent. Their phenotypic characteristics are described in more detail in Chapter IV.

Finally, the stability of pWX9 cannot be assumed to be due solely to the presence of the pSC101 par function, since copy number analysis shows that pWX9 increased in copy number with increasing generations of

chemostat culture (Fig.3.13).

3.4 Destabilization of plasmid pDS1109

3.4.1 Introduction

The segregational stability of two ColE1 derivatives encoding ampicillin resistance, pDS1109 and RSF2124, has been tested by others employing chemostat culture. These plasmid derivatives are identical except for the location of Tn1 inserts (Fig.3.14). Jones et al. (1980b) found that pDS1109 was stably maintained during chemostat culture, suggesting that ColE1 may encode a particular mechanism for ensuring the fidelity of plasmid segregation at cell division. Adams et al. (1979) found that RSF2124 was also stably maintained during chemostat culture, except when DNA was inserted at the EcoRI site. In addition, Jones et al. (1980a) found that RSF2124 derivatives, with fragments of foreign DNA also inserted at the EcoRI site, were not stably maintained during chemostat culture. These results suggest that there may reside a function in the region of the EcoRI site, distinct from that of colicin E1 expression, which is essential for the fidelity of plasmid segregation at cell division. If this is so, then pDS1109 should also be destabilized by insertion of foreign DNA at the EcoRI site.

3.4.2 Results

Confirmation of this was obtained by inserting fragments of lambda DNA, derived from essentially

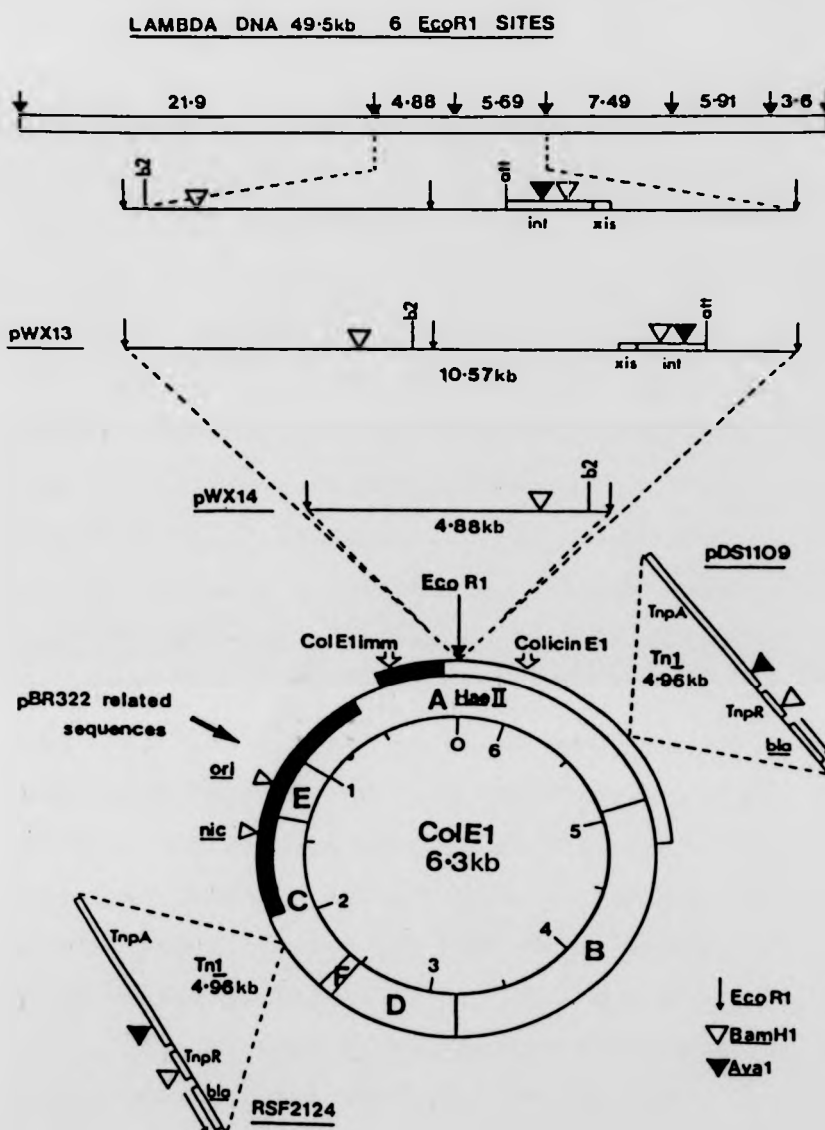


Figure 3.14

Composite illustration of plasmid ColE1 derivatives generated by either Tn1 insertion (pDS1109 & RSF2124), or by the insertion of lambda EcoRI fragments (pWX13 & pWX14).

'silent' regions of the lambda genome, at the EcoRI site of pDS1109 (Fig.3.14). The cloning procedure involved isolation and purification of pDS1109 DNA from strain W5445, following chloramphenicol amplification and CsCl-EtBr density gradient centrifugation. EcoRI restricted preparations of this DNA were mixed and ligated with EcoRI digested lambda cI857Sam7 DNA, obtained from a commercial source (Bethesda Research Laboratories (U.K.) Ltd., Cambridge, England), (Fig. 3.15). The ligation mixture was used to transform strain W5445 to ampicillin resistance. Rapid, small scale plasmid analysis of eight ampicillin-resistant transformants indicated the presence of three probable recombinants (Fig.3.16 A), one large recombinant and two co-migrating lower molecular weight recombinants. Plasmid DNA from the larger, and one of the small recombinants, was isolated and purified following chloramphenicol amplification and CsCl-EtBr density gradient centrifugation (Fig.3.16 B, lanes b & e respectively). Restriction digest analysis revealed that the larger recombinant contained two lambda cI857Sam7 EcoRI generated fragments of 4.8kb and 5.6kb in size, while the smaller recombinant contained only a 4.8kb fragment (Fig.3.17, lanes e & k respectively). The larger recombinant plasmid was designated pWX13 and the smaller pWX14.

Location of the lambda DNA fragments was confirmed by HaeII restriction analysis (Fig.3.17). HaeII digestion of pDS1109 DNA generates six observable fragments, following agarose gel electrophoresis

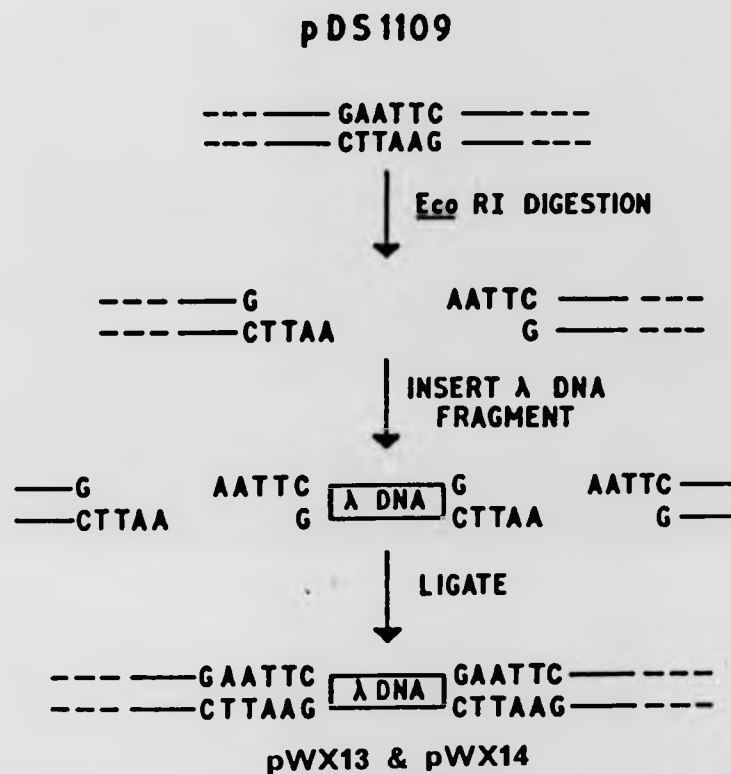
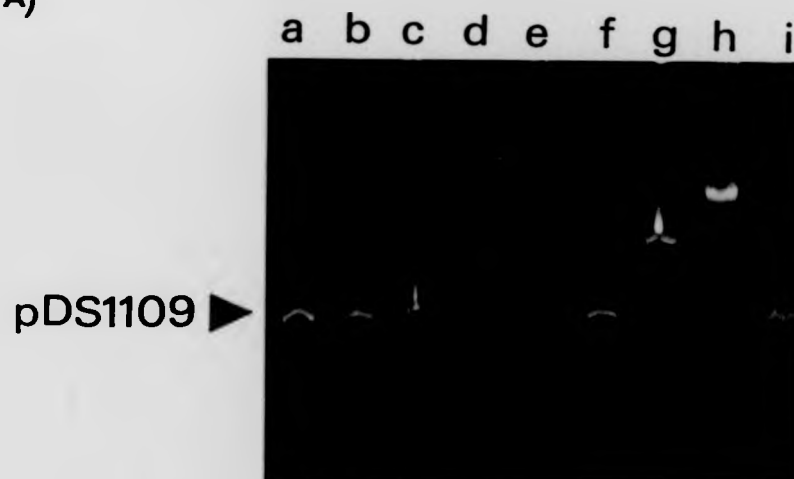


Figure 3.15

Construction of plasmids pWX13 and pWX14 by insertion of EcoRI generated fragments of lambda DNA into the unique EcoRI site of pDS1109.

(Reproduced from Primrose et al., 1984).

A)



B)

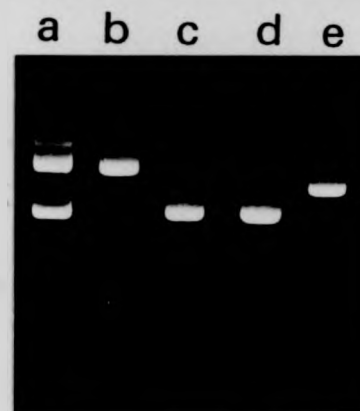


Figure 3.16

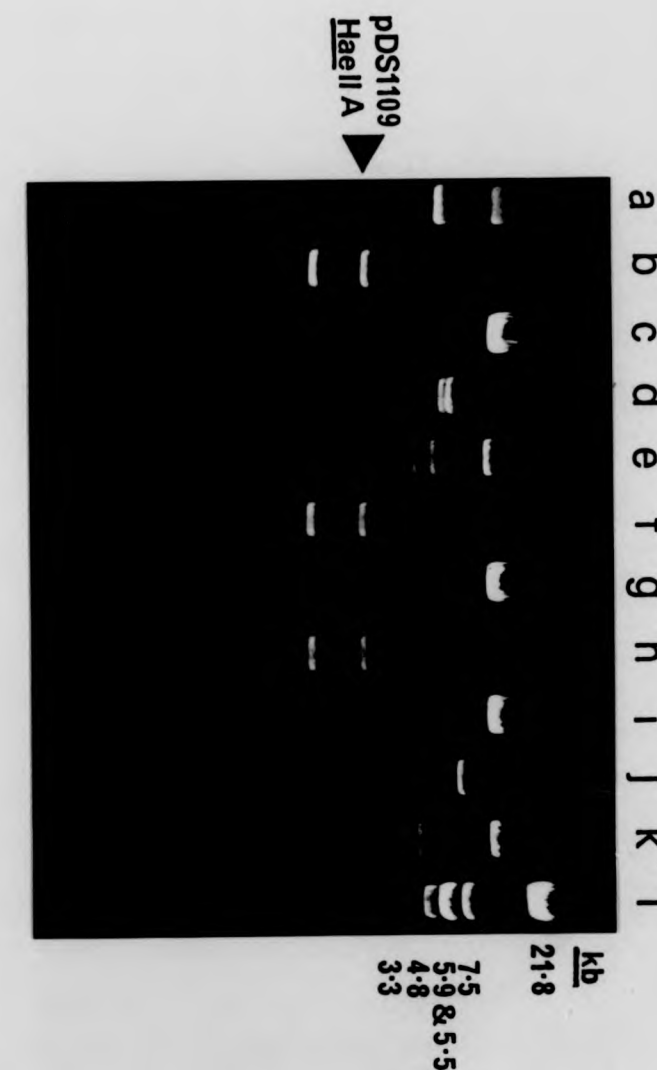
Agarose gel electrophoresis of presumptive clones of *E.coli* strain W5445, bearing plasmid recombinants of pDS1109 containing lambda *Eco*RI DNA inserts.

A) Agarose gel electrophoresis of rapid, small-scale plasmid preparations obtained from eight ampicillin-resistant transformants of strain W5445, lane a) plasmid pDS1109, lanes b to i) rapid plasmid preparations from strain W5445 ampicillin-resistant transformants.

B) Agarose gel electrophoresis of purified plasmid DNA preparations obtained from four ampicillin-resistant transformants of strain W5445 harvested from CsCl-EtBr density gradients, lane a) pDS1109 DNA, b) pWX13 DNA, c) and d) pDS1109/1 and pDS1109/2 DNA and, e) pWX14 DNA.

Figure 3.17

Comparative restriction endonuclease analysis of plasmids pDS1109, pDS1109/1 and /2, pWX13 and pWX14. Lane a) undigested pDS1109 DNA, b and c) HaeII and EcoRI digests respectively of pDS1109 DNA, d and e) HaeII and EcoRI digests respectively of pWX13 DNA, f and g) HaeII and EcoRI digests respectively of pDS1109/2 DNA, h and i) HaeII and EcoRI digests respectively of pDS1109/1 DNA, j and k) HaeII and EcoRI digests respectively of pWX14 DNA, e) EcoRI digest of lambda DNA.



(Fig.3.17, lane b). The largest of these fragments contains the unique EcoRI site of pDS1109, therefore, HaeII digests of pWX13 and pWX14 DNA results in the absence of this HaeII fragment (Fig.3.17, lanes d & j respectively), and the appearance of higher molecular weight fragments due to covalent attachment of lambda DNA. The 4.8kb fragment derived from lambda in pWX14 does not contain a HaeII restriction site, indicating that the 5.6kb lambda fragment in pWX13 does contain a single HaeII site (Fig.3.17, lane d). Further characterisation of plasmids pWX13 and pWX14 was carried out using restriction analysis, in conjunction with published restriction data relating to lambda, ColE1 and Tn3 (Tn1) sequences (Sanger, *et al.*, 1982; Oka, *et al.*, 1979; Heffron, *et al.*, 1979).

Tn3 contains a single BamHI restriction site. BamHI restriction analysis of pDS1109, indicates that this restriction site is absent within the ColE1 sequences of pDS1109 (Fig.3.18 A, lane e). Insertion of Tn3 into ColE1, generating pDS1109, has been reported to occur within the colicin structural gene at a location about 0.9kb from the unique EcoRI site (Dougan & Sherratt, 1977), (Fig.3.14). Restriction analysis of pDS1109 DNA with an EcoRI/BamHI double digest, confirmed the orientation of the Tn3 insert in pDS1109 with respect to the EcoRI site (Fig.3.18 A, lane f). Likewise, since the 4.8kb lambda EcoRI fragment insert contains a single BamHI site, BamHI restriction analysis of pWX14 determined the orientation of this EcoRI inserted fragment relative to the Tn3 insertion (Fig.3.18 A,

Figure 3.18

A) Comparative restriction endonuclease analysis of plasmids pDS1109 and pWX14. Lane a) HindIII digest of lambda DNA, b) EcoRI digest of pDS1109 DNA, c) AvaI digest of pDS1109 DNA, d) undigested pDS1109 DNA, e) BamHI digest of pDS1109 DNA, f) BamHI/EcoRI double digest of pDS1109 DNA, g) undigested pWX14 DNA, h) BamHI digest of pWX14 DNA, i) EcoRI/HindIII double digest of lambda DNA.

B) Restriction endonuclease analysis of plasmid pWX13. Lane a) undigested pWX13 DNA, b) BamHI digest of pWX13 DNA, c) EcoRI/HindIII double digest of lambda DNA.

B) a b c



kb

21.8

5.2 & 5.0

4.2

3.3

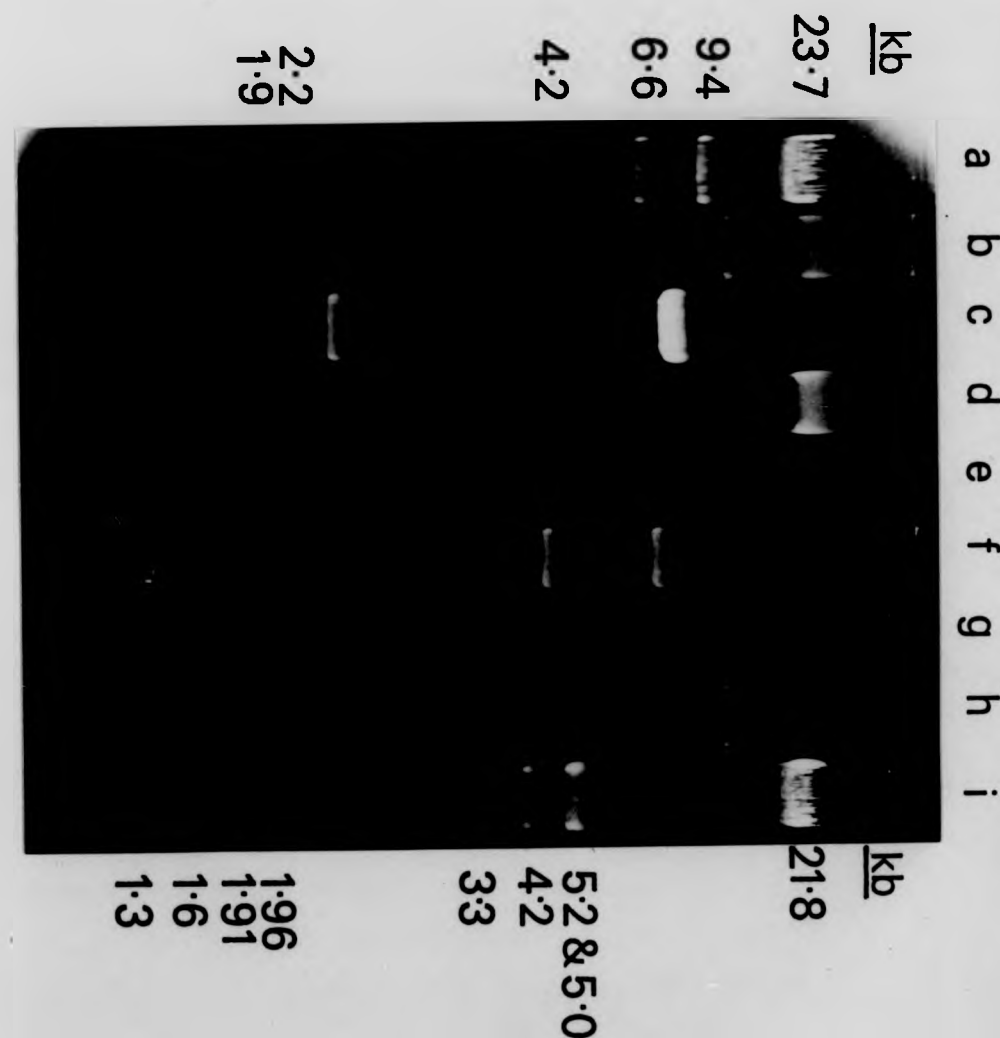
1.96

1.90

1.6

1.3

A)



kb

23.7

9.4

6.6

4.2

2.2
1.9

kb

21.8

5.2 & 5.0

4.2

3.3

1.96
1.91

1.6

1.3

lane h). Similarly, the 5.6kb lambda EcoRI fragment also contains a single BamHI site. BamHI restriction analysis of pWX13 (Fig.3.18 B), not only determined the orientation of the 4.8kb and 5.6kb fragment inserts with respect to one another, but also their orientation relative to the Tn3 insertion.

Neither plasmid pWX13 nor pWX14 was stably maintained during phosphate-limited chemostat culture (Fig.3.19). However, the two plasmids did not segregate at the same rate, the larger plasmid pWX13, segregated more rapidly than the smaller plasmid pWX14. In addition, cells from both chemostat experiments, when plated directly onto rich medium, did not exhibit any marked differences in their colony morphologies as observed during previous chemostat experiments.

3.4.3 Concluding remarks

The above evidence would seem to suggest that the unique EcoRI site of plasmid ColE1, may lie within a region responsible for the segregation of this plasmid at cell division. However, as alluded to in similar foregoing experiments several complicating factors make this assumption unlikely.

Although plasmids pWX13 and pWX14 are similarly constructed derivatives of pDS1109, they nevertheless showed markedly different segregational stabilities during chemostat culture (Fig.3.19). Since an inverse relationship has been found to occur between the size of some ColE1 derivatives and their copy number (Dougan & Sherratt, 1977; Kasner et al., 1985), DNA inserts of

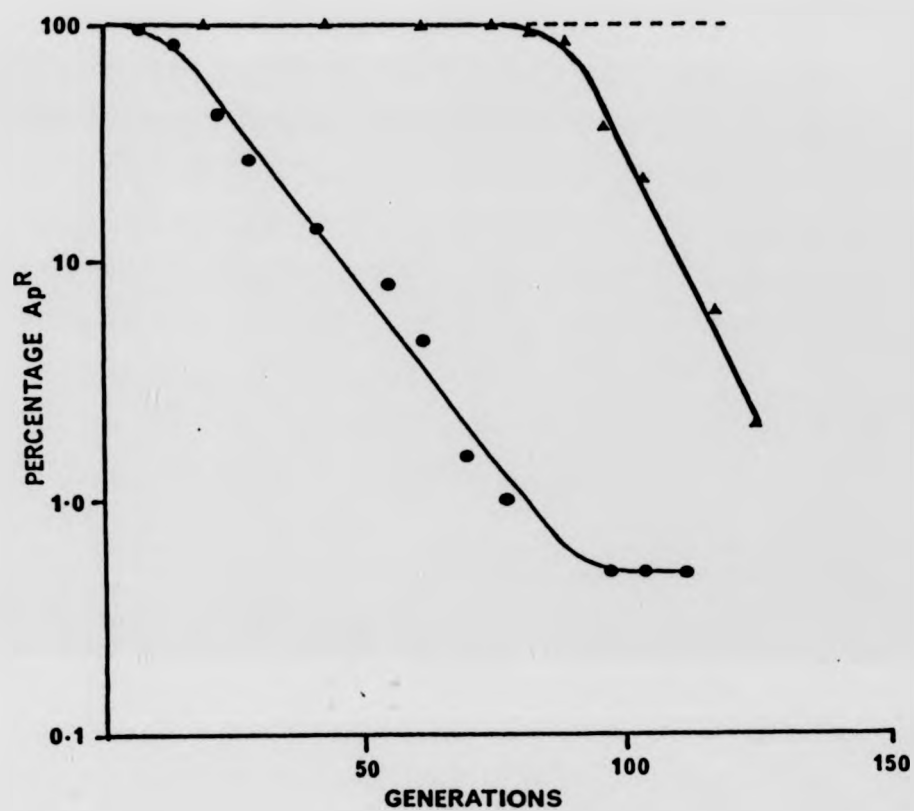


Figure 3.19

Persistence of plasmids pWX13 and pWX14 in strain W5445 during phosphate-limited chemostat culture. Results are presented as a semi-logarithmic plot of percentage ampicillin-resistant population with number of generations; ●—●, pWX13; ▲—▲, pWX14.

Data showing the persistence of plasmid pDS1109 in *E.coli* strain W5445 during phosphate-limited chemostat culture;-----, is taken from Jones *et al.* (1980b). In all three experiments the dilution rate was 0.2hr^{-1} (a mean generation time of about 3.47hrs.).

increasing size may have been expected to result in a decreased copy number in the order of pDS1109 > pWX14 > pWX13. Jones *et al.* (1980b) have inferred that pDS1109 is inherently stable. This assumption was based on the finding that a 5-fold reduction in the relative plasmid DNA content of pDS1109-bearing cells, occurred during glucose limitation (Fig.1.7B). This reduction in plasmid DNA content appeared to level out at about 100 generations, subsequent chemostat culture was only continued for a further 20 generations. If this 5-fold decrease in the relative plasmid DNA content of pDS1109 was a reversal of a 5-fold increase in plasmid copy number, occurring during initial batch growth to stationary phase (B.Polisky, personal communication, Indiana University, Bloomington, Indiana. 1982), then pDS1109 may have shown segregational stability during glucose limitation simply as a consequence of a sufficiently high copy number. Assuming that pWX14 has a copy number marginally less than pDS1109, then the kinetics of appearance of pWX14-free segregants would seem to imply that the stability of pDS1109 is related to its copy number. Furthermore, the presence of Tn1-derived sequences does not appear to confer a selective advantage on pWX14-bearing cells during phosphate limitation, as shown for other transposable genetic elements during nutrient-limited chemostat culture (Hartl *et al.*, 1983; Chao *et al.*, 1983).

The instability of pWX13, however, appears greater than that attributable to a simple postulated reduction in copy number. The generation of pDS1109 derivatives

possessing essentially 'silent' inserts of DNA at a unique restriction site, was an essential aim of the cloning procedure. In the case of pWX13, two lambda-derived EcoRI fragments were inserted, one of these fragments specifies the lambda prophage integration and excision system (Fig.3.14). Enhanced segregational instability of this pDS1109 derivative, may have been due to increased recombination mediated by the lambda-derived prophage integration and excision system. Indeed, phosphate-limited growth may be expected to lead to an induction of the host-integration factor himA (Wanner, 1983), which is required for lambda genome integration and excision. Glucose, in contrast to phosphate limitation, may alternatively lead to a greater degree of segregational stability for pWX13-bearing cells.

It would appear, therefore, that one factor which may contribute to the segregational stability of pDS1109 (and therefore ColE1), during chemostat culture, is a sufficiently high plasmid copy number. However, this does not resolve the question as to whether an additional stabilizing factor is located within the unique EcoRI region of ColE1.

3.5 Segregational stability of a pBR322 derivative carrying the HaeIIA fragment of plasmid pDS1109

3.5.1 Introduction

Results presented previously have suggested that the segregational instability of pBR322 may be rectified

by the insertion of a par function derived from an unrelated plasmid. This would indicate that similar plasmid-encoded stability functions could be likewise isolated and identified.

3.5.2 Results

The EcoRI site of plasmid ColEl lies within the HaeIIA fragment. Since previous results implicate the HaeIIA fragment as containing a region which may be essential to the segregational stability of ColEl, and since portions of the HaeIIA sequence are absent or nonfunctional in pBR322 (Fig.1.9), it was of interest to determine what effects on stability this region may have when cloned into pBR322. However, if the entire HaeIIA fragment were cloned it may confer on the recombinant an ability to synthesize colicin El. It was for this reason that I.M. Jones of this laboratory, consequently cloned the residue of the ColEl:HaeIIA fragment from pDS1109. (The cloning procedure involved a partial HaeII digestion of pBR322, and this DNA was then mixed and ligated with a total HaeII digest of pDS1109, the ligation mixture was used to transform strain W5445 to ampicillin resistance. Since the HaeIIA fragment encodes immunity to colicin El, selection was made for colicin El-immune and tetracycline-sensitive recombinant plasmids. One such plasmid generated was designated pWX11), (Fig.3.20). Using EcoRI and HaeII restriction analysis, confirmation was obtained of the presence, location and orientation, of the ColEl:HaeIIA residual fragment from pDS1109 in pWX11 (Fig.3.21).

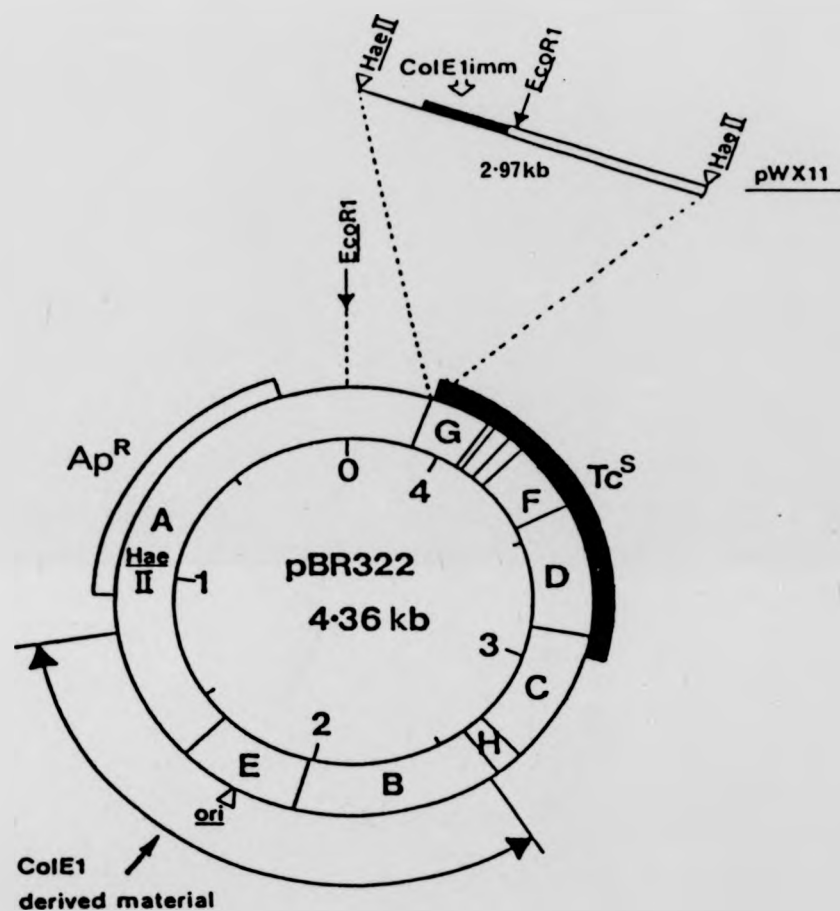


Figure 3.20

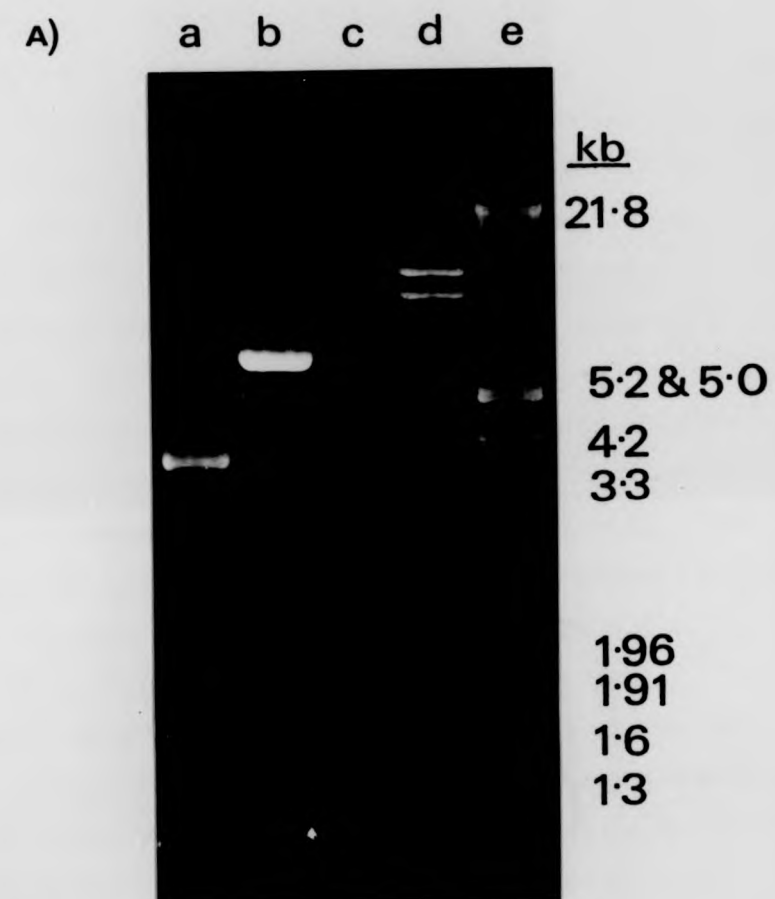
Illustration of recombinant pBR322 plasmid pWX11. (The extent of any small *HaeII* restriction digest generated deletion(s), within the tetracycline resistance coding region of pBR322 were not determined).

Figure 3.21

Restriction endonuclease analysis of plasmid pWX11, together with confirmation of the structure of plasmid pNT3, following comparative restriction endonuclease analysis of plasmids pDS1109 and pNT3. (see Fig.3.24).

A) Lane a) undigested pWX11 DNA, b) EcoRI digest of pWX11 DNA, c) undigested pWX13 DNA, d) BamHI digest of pWX13 DNA, e) EcoRI/HindIII double digest of lambda DNA.

B) Lane a) undigested pDS1109 DNA, b) HaeII digest of pDS1109 DNA, c) undigested pNT3 DNA, d) HaeII digest of pNT3 DNA, e) undigested pWX11 DNA, f) blank lane, g) HaeII digest of pWX11 DNA, h) undigested pWX11 DNA.



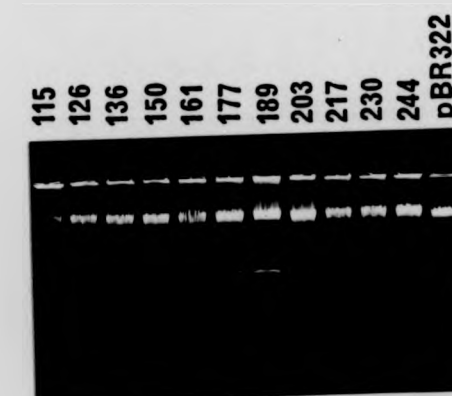
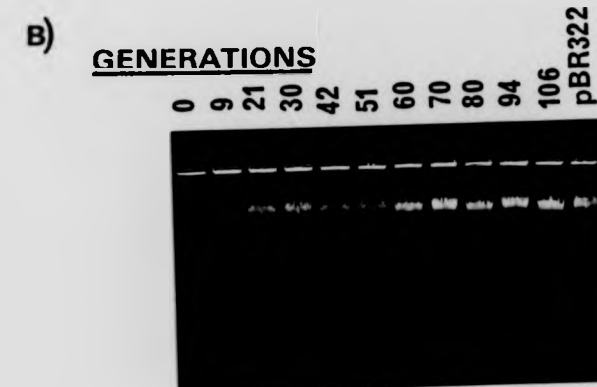
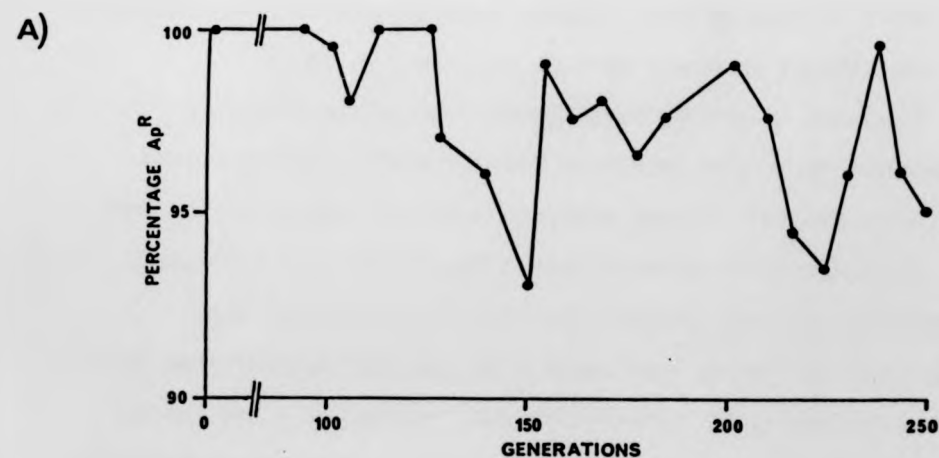
Plasmid pWX11 was stably maintained for 100 generations of phosphate-limited chemostat culture (Fig.3.22 A), and as demonstrated by analysis in agarose gels, did not appear to undergo any detectable structural deletion, or insertion rearrangements (Fig.3.22 B). However, after 100 generations, pWX11-free segregants arose and persisted as an approximately 3% sub-population, in an analogous manner to the chemostat cultures of pWX9-bearing cells. In addition, similar colony morphology differences, to those occurring during the chemostat culture of pWX9-bearing cells, were also observed following the plating of cells directly from the chemostat onto rich medium. No correlation appeared to exist between colony morphology and ampicillin resistance or sensitivity. These plasmid-free segregants, like those isolated during the chemostat culture of pWX9-bearing cells, also represent mutants of the host strain W5445. Their phenotypic characteristics are described in more detail in Chapter IV.

Finally, plasmid copy number determination of chemostat cultured cells carrying pWX11, showed that pWX11 exhibited a copy number fluctuating at about 100, for at least 100 generations (Fig.3.23). A marginal reduction in copy number during this period, was completed at about the same time as the appearance of plasmid-free host variant cells. From this period to about 160 generations, plasmid-copy number progressively increased to approximately 140, and persisted at this level for a further 60 generations, at which time the chemostat experiment was ended.

Figure 3.22

A) Persistence of plasmid pWX11 in *E.coli* strain W5445 during phosphate-limited chemostat culture. Results are presented as percentage ampicillin-resistant population with number of generations. The dilution rate was 0.2hr^{-1} (a mean generation time of about 3.47hrs.).

B) Agarose gel electrophoresis of whole-cell lysates obtained from samples directly harvested during chemostat culture of pWX11. These were subsequently used for the determination of plasmid copy number (See Fig.3.23).



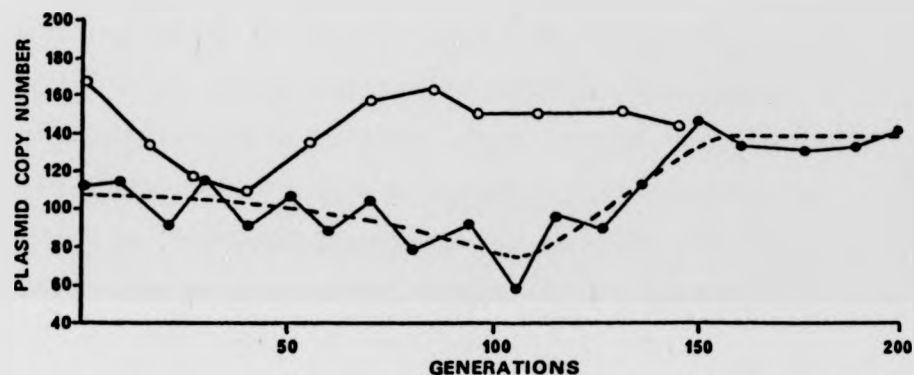


Figure 3.23

Changes in the copy number of plasmids pWX11 and pNT3, when plasmid-containing cells of *E.coli* strain W5445 were grown in phosphate-limited chemostats. ●—●, pWX11 and ○—○, pNT3, the overall trend in copy number variation is indicated by;-----.

3.5.3 Concluding remarks

The persistence of pWX11-bearing cells during phosphate limitation, and the isolation of host mutants similar to those observed during continuous culture of pWX9-bearing cells, poses two distinct questions.

The first of these relates to whether the HaeIIA fragment, derived from pDS1109, does indeed specify a stability function within the unique EcoRI region. As with previous chemostat experiments, carried out to investigate the segregational stability of pBR322 derivatives, the elevated copy number of pWX11, determined during chemostat culture (Fig.3.23), does not allow this possibility to be resolved.

The HaeIIA fragment of pDS1109 contains within it the promoter region and structural gene for RNAI of ColEI, as well as the promoter region and target sequence of RNAII (Fig.1.3). ColEI-derived expression of RNAI and its target sequence within RNAII, would be expected to titrate pBR322-derived expression of the Rop protein, leading to an increase in pWX11 copy number. Any negative effect on the control of plasmid replication initiated at the pBR322 origin, by ColEI-derived expression of RNAI, may be marginal, since the RNAI molecules of ColEI and pBR322 are known to differ slightly (Hoshimoto-Gotoh & Timmis, 1981). In addition, the insertional inactivation of the tetracycline resistance coding region may also have been expected to contribute to an increase in plasmid copy number (Stueber & Bujard, 1982), as well as removing any

growth-rate disadvantage associated with the expression of tetracycline resistance (Lee & Edlin, 1985). Furthermore, if certain ColE1 sequences confer a selective disadvantage that outweighs any selective advantage conferred by Tn₁, then such an advantage may not be observable in the presence of ColE1. Indeed, a selective disadvantage does appear to be conferred on cells bearing plasmids pWX14 (Fig.3.19), or pDS1109 (Fig.1.7A), (Jones et al., 1980b). Therefore, as a consequence of the presence of Tn₁-derived sequences, a small, but significant selective advantage may have been conferred on pWX11-bearing cells. However, pWX11-bearing cells of strain W5445 appear to be selectively neutral, even in the presence of plasmid-free host mutant cells.

The second question posed relates to the nature of the selective pressure which gives rise to plasmid-free host mutants, seemingly defective in plasmid segregation. It is notable that during chemostat culture of both pWX9- and pWX11-bearing cells at a dilution rate of 0.2hr^{-1} , the detection of plasmid-free host mutants occurred at about 100 generations (Fig.3.10 A & 3.22 A). This may suggest that the mutational basis for the generation of such mutants is the occurrence of periodic selection, possibly conforming to the 'copy-error' hypothesis. Since both pWX9 and pWX11 exhibited a copy number, during chemostat culture, several-fold greater than pBR322 (Fig.3.13 & 3.23), then one possible explanation could be that such high plasmid copy numbers may exact intracellular limitations,

conferring a selective pressure which gives rise to plasmid-free host mutants defective in plasmid segregation. It is interesting to note that plasmid-free segregants arising during chemostat culture of pWX9-bearing cells, were less viable than those obtained during chemostat culture of pWX11-bearing cells. All but one of the plasmid-free segregants obtained during chemostat culture of pWX9-bearing cells, failed to survive more than about two days on rich medium. It would appear, therefore, that detection of similar host mutant plasmid-free segregants, may or may not occur, as a consequence of not only the resident plasmid and possibly its copy number, but also experimental parameters such as dilution rate, temperature and nutritional plating conditions.

3.6 Segregational stability of plasmids pNT3 and pAT153 during chemostat culture

3.6.1 Introduction

Ohmori & Tomizawa (1979) have constructed a series of mini-ColE1 derivatives. The segregational stability of the smallest of these, pNT3, was assayed in an attempt to determine to what extent the region surrounding the EcoRI site of ColE1 may be essential for the postulated fidelity of plasmid segregation. pNT3 consists of the entire HaeIII fragment of ColE1, together with correctly orientated adjacent portions of the HaeIIA and C fragments linked to Tn3 (Fig.3.24). The partial HaeIIA fragment terminates at about 684bp

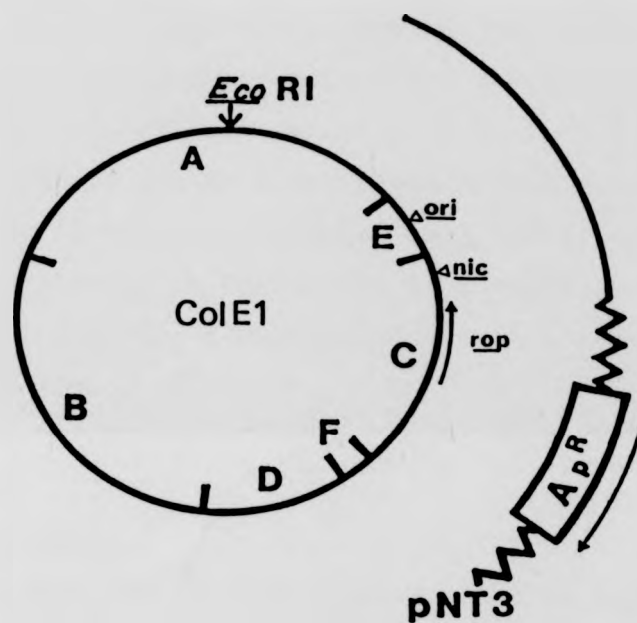


Figure 3.24

HaeII restriction map of plasmid ColE1 and the structure of plasmid pNT3. (Ohmori & Tomizawa, (1979), see also Fig.3.21 B).

from the replication origin (Ohmori & Tomizawa, 1979). The HaeIIC fragment of ColE1 encodes the Rop protein, a trans-acting negative regulatory element that affects the initiation of ColE1 DNA replication (Cesareni et al., 1982). Absence of Rop results in a 2- to 5-fold increase in ColE1 or pBR322 copy number (Davison, 1984). Jones & Melling (1984) have reported that pAT153, a HaeIIB and H rop deletion derivative of pBR322, is stably maintained during a variety of nutrient-limited chemostat cultures.

3.6.2 Results

a) Plasmid pNT3

Since pNT3 does not possess the surrounding EcoRI region of ColE1, it may be expected that pNT3 would not be maintained during conditions of phosphate limitation. However, during 200 generations of phosphate-limited chemostat culture, no plasmid-free segregants of W5445 carrying pNT3 were detected (Fig.3.25 A). However, colony morphology differences similar to those deriving from the chemostat cultures of pWX9- and pWX11-bearing cells were observed. Agarose gel analysis indicated that no major structural deletion, or insertion rearrangement of pNT3 had occurred during chemostat culture (Fig.3.25 B). Plasmid copy number determination, of chemostat-cultured cells carrying pNT3, showed that pNT3 exhibited an elevated copy number several-fold greater than pBR322 or ColE1 (Fig.3.23). A marked reduction in plasmid-copy number occurred during the first 40 generations. However, this copy number

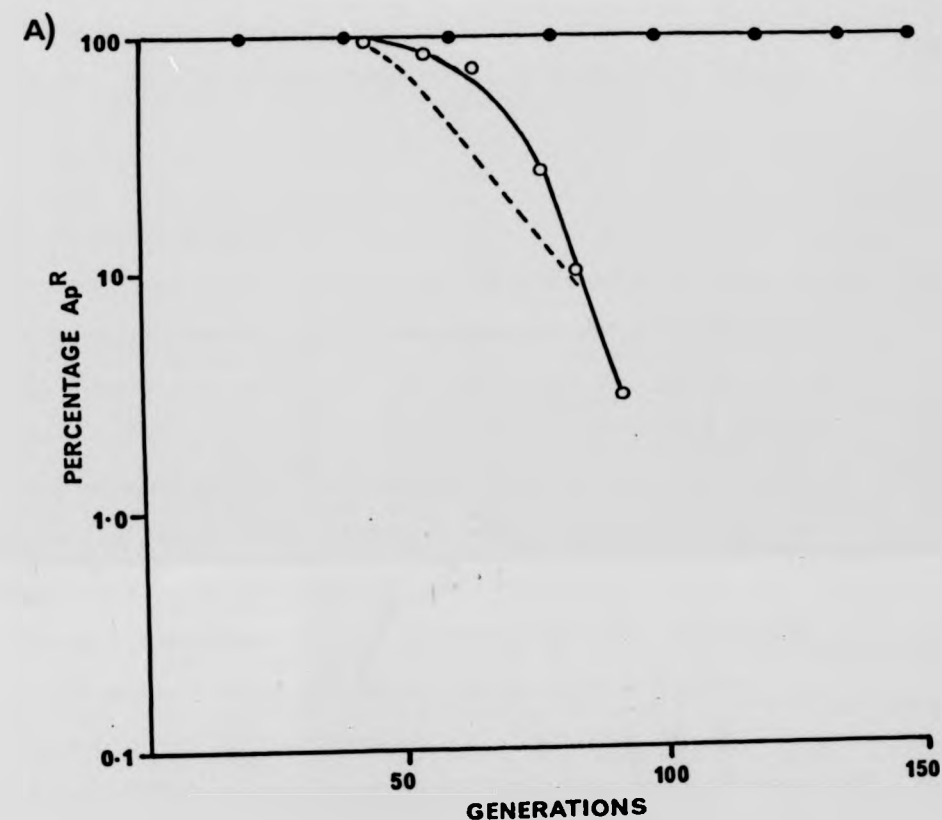
Figure 3.25

A) Persistence of plasmid pNT3 during phosphate-limited chemostat culture and pAT153 during glucose-limited chemostat culture, in *E.coli* strain W5445. Results are presented as semi-logarithmic plots of percentage ampicillin-resistant population with number of generations; ●—●, pNT3 ; ○—○, pAT153.

Data showing the persistence of plasmid pBR322 in *E.coli* strain W5445 during phosphate-limited chemostat culture;-----, is taken from Jones *et al.* (1980b).

The dilution rate during all three chemostat experiments was 0.2hr^{-1} (a mean generation time of about 3.47hrs.).

B) Agarose gel electrophoresis of whole-cell lysates obtained from samples, directly harvested during chemostat culture of pNT3. These were subsequently used for the determination of plasmid copy number (See Fig.3.23).



reduction was corrected over a subsequent 40 generation period, to give a final average copy number of about 160.

b) Plasmid pAT153

The segregational stability of pNT3 may be due to an elevated copy number, as a consequence of the absence of the rop function. However, in contrast to the findings of Jones & Melling (1984) strain W5445 carrying pAT153 produced plasmid-free segregants during glucose-limited chemostat culture (Fig.3.25 A). When compared to the segregational instability of pBR322 during phosphate-limitation, it is evident that an increased number of generations elapsed before plasmid-free segregants began to predominate. Finally, no colony morphology differences were observed when pAT153-bearing cells, under glucose limitation, were plated directly onto rich medium.

3.6.3 Concluding remarks

Chemostat culture of pNT3 represented one of three experiments aimed at resolving the question as to whether ColE1 encodes a postulated stability function, necessary for the fidelity of plasmid segregation at cell division. As with previous experiments examining the segregational stability of ColE1-type plasmid derivatives during chemostat culture, no conclusion with respect to ColE1 segregation could be inferred, as a consequence of the high copy number of pNT3 (Fig.3.23). This observed high copy number for pNT3 may to some

extent be attributable to the absence of the Rop protein, however, correctly orientated transcriptional readthrough from the beta-lactamase gene of Tn₃, into the region of ColE1 responsible for plasmid replication (Fig.3.24), may have altered the ratio of RNAI to RNAII, resulting in a further increase in plasmid copy number. Indeed, an almost identical ColE1-derived plasmid, pDM56642, has been shown to exhibit a 4-fold increase in copy number, primarily as the result of a similar transcriptional readthrough (Schmidt & Inselburg, 1982).

The results obtained for plasmid pAT153 confirm the segregational instability of this plasmid in strain W5445. This host strain, in contrast to that used by Jones & Melling (1984) is not deficient in host recombinational pathways. Hence, the segregational instability attributable to pAT153-bearing cells of strain W5445, may be due to an increase in plasmid multimerization. Absence of the Rop protein results in an increase in the copy number of pAT153 (Davison, 1984). Detection of pAT153-free cells following a number of generations beyond those necessary for the detection of pBR322-free cells, under similar chemostat conditions, would appear to be due to the relative difference in plasmid copy numbers of pAT153 and pBR322. Expression of tetracycline resistance, would in addition, be expected to confer a similar selective disadvantage on pAT153-bearing cells, as is the case for pBR322-bearing cells (Lee & Edlin, 1985), leading to similar kinetics for the appearance of plasmid-free segregants (Fig.3.25).

Finally, the characteristic differences in colony morphology of pNT3-bearing cells, when plated directly from the chemostat onto rich medium, may suggest that mutants, similar to plasmid-free mutants arising during chemostat culture of pWX9- and pWX11-bearing cells, may also have arisen during the chemostat culture of pNT3-bearing cells. However, such postulated mutants do not appear to have given rise to plasmid-free segregants. In contrast, colony differences during chemostat culture of pAT153-bearing cells were not observed, which may be a reflection of either the nature of the plasmid and its copy number, or the use of a carbon limitation.

3.7 Discussion

3.7.1 Introduction

Original concepts relating to the segregational stability of ColE1-type plasmids during chemostat culture have been outlined by Jones et al. (1980b). These workers established that plasmids pBR322 and pDS1109 do not confer a selective advantage. Plasmid pBR322 was shown to exhibit segregational instability, while pDS1109, although undergoing a 5-fold reduction in relative plasmid DNA content, was shown to be segregationally stable during chemostat culture. It was subsequently postulated that plasmid ColE1 may encode a par function. However, a more complicated picture now emerges regarding the segregational stability of ColE1-type plasmids during chemostat culture.

3.7.2 Copy number, reproductive fitness and ColE1-type plasmid stability

Plasmid copy number is undoubtedly an important parameter when considering segregational plasmid stability. Indeed, in a recent theoretical study it was suggested that plasmids which exhibit a copy number of twenty or more, may be expected to be stably maintained for at least 100 generations (Seo & Bailey, 1985). Not surprisingly, therefore, plasmids pNT3, pWX11, pOU93 and pWX9 are stably maintained (Fig.3.10;3.22 & 3.25), since copy number analysis shows these plasmids to exhibit a copy number, during chemostat culture, several-fold greater than pBR322 (Fig.3.13 & 3.23).

All of the above stably maintained plasmids were cultured under identical chemostat conditions (i.e., phosphate limitation, a dilution rate of 0.2hr^{-1} and a temperature of 37°C), which may explain why each of the pBR322 derivatives (pOU93, pWX9 & pWX11), exhibited a copy number of about 100 at the commencement of their respective chemostat cultures. This appears to conform to about a 4-fold increase in plasmid copy number, similar to that experienced by pBR322 upon transition from exponential to stationary phase of batch culture (Stueber & Bujard, 1982).

In contrast, the mini-ColE1 plasmid, pNT3, exhibited an initial copy number of about 170. pNT3, however, may not encode the Rop polypeptide, which is presumably expressed by the pBR322 derivatives. Absence of the Rop

protein results in a 2- to 5-fold increase in the copy number of ColE1 (Davison, 1984). ColE1 is reported to have a copy number of about 20 (Veltkamp & Stuitje, 1981), therefore, a ColE1 rop⁻ derivative may be expected to have a copy number of the order of 60. Transition from the exponential to stationary phase of batch culture, might be expected to elevate this copy number some 5-fold (B.Polisky, personal communication, Indiana University, Bloomington, Indiana, 1982), resulting in a final copy number of about 300. In addition, a postulated transcriptional readthrough from the beta-lactamase gene of Tn3 may also have been expected to boost copy number (Schmidt & Inselburg, 1982). However, it is evident that pNT3 failed to attain such a high plasmid copy number. An initial copy number of 170, and final copy numbers for pNT3 (about 160), pWX11 (about 140) and pWX9 (about 290), (Fig.3.13 & 3.23), all appear to correlate with the size in kilobase pairs of the respective plasmids, giving final plasmid DNA contents per genome equivalent of 929kb for pNT3, 1044kb for pWX11 and 962kb for pWX9. Hence, it appears that the initial and final copy number for pNT3 corresponds to a plasmid DNA content maximal for E.coli strain W5445, under the prevailing chemostat culture conditions.

Previous work from this laboratory has suggested that ColE1-type replicons undergo a reduction in copy number, as a consequence of nutrient-limited chemostat culture (Jones et al., 1980b). However, a similar reduction in the copy number of plasmids pNT3,

pWX11, pOU93 and pWX9 during chemostat culture, did not occur, although initial reductions in the copy number of plasmids pNT3 and pWX11 were observable (Fig.3.13 & 3.23). For high copy number plasmids that are randomly distributed to daughter cells, a more important parameter may be that of reproductive fitness. Expression of pBR322-encoded tetracycline resistance has been shown to confer a selective disadvantage on pBR322-bearing cells (Lee & Edlin, 1985). Notably, inactivation of tetracycline resistance resulted in plasmid-bearing cells possessing no greater growth-rate disadvantage than plasmid-free cells, indicating that at least during glucose limitation, maintenance of a pBR322 copy number may not in itself confer a growth disadvantage.

Therefore, observations relating to a reduction in the copy numbers of pDS1109 (Jones et al., 1980b), and pBR322 (Wouters et al., 1980), during nutrient-limited chemostat culture, may have been a consequence of the expression of a reproductive disadvantage proportional to plasmid copy number. This would suggest that some factor(s), expressed by pDS1109, confers a selective disadvantage. In support of this assertion, Helling et al. (1981), have also shown that RSF2124 affects the growth-rate of host cells during nutrient-limited chemostat culture. The selective disadvantage conferred by plasmids pDS1109 or RSF2124, may be specified by either the ColE1 or Tn1 sequences. Certain mobile genetic elements are known to confer a selective advantage, however, at a high-copy number they may also confer a selective disadvantage (Hartl et al., 1983).

Since a plasmid copy number reduction similar to that observed for pDS1109 (Jones *et al.*, 1980b), did not occur during chemostat culture of pWX11- or pNT3-bearing cells (Fig.3.23), then the selective disadvantage is presumably specified within ColE1 sequences, other than those present on plasmids pWX11 or pNT3.

However, a decrease in pNT3 copy number, during the first 40 generations of chemostat culture, may reflect a growth disadvantage as a consequence of the random distribution of a high number of plasmid copies at cell division. Those cells of the population inheriting a copy number greater than that of the population mean, may have components of their host DNA synthesizing machinery sequestered by plasmid replication origins, which are themselves, unable to initiate plasmid replication. This may confer a marginal selective advantage on cells inheriting a copy number less than that of the mean, leading to a concomitant decrease in the mean plasmid copy number distribution for the chemostat population with increasing number of generations. A reduction, therefore, in the mean distribution of plasmid copy number within a chemostat population, may be expected to continue to a level at which only a marginal effect is exerted on the competitiveness of plasmid-bearing cells.

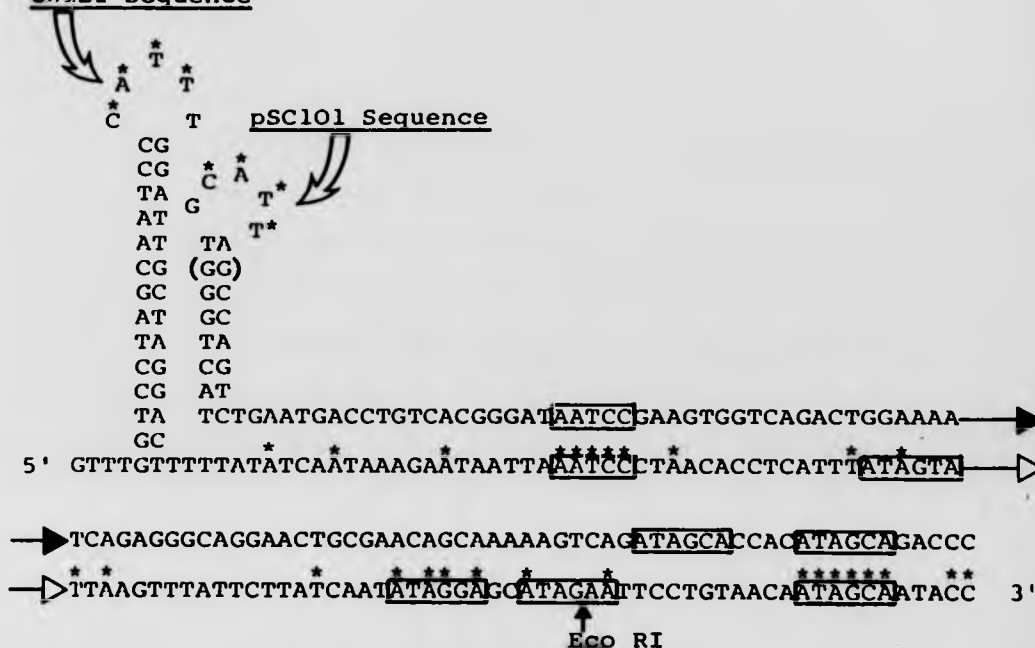
The postulated existence of a par function, presumed to be encoded within the region surrounding the unique EcoRI site of ColE1, could not be substantiated. A comparison of the DNA sequence surrounding the EcoRI

site of ColE1 (Oka *et al.*, 1979), with that of the par region of pSC101 (Miller *et al.*, 1983), indicates that no significant sequence homology exists between the two regions (Fig.3.26). The only structural similarity is the presence of a hairpin loop. The ColE1-encoded stem and loop structure has been shown to occur in supercoiled plasmid DNA (Panayotatos & Wells, 1981). However, the stems are of different size and sequence, and may function as transcriptional termination signals.

It would appear, therefore, that the segregational stability of pDS1109 is primarily due to a high plasmid copy number, as may be suggested by the segregational instability of pWX14-bearing cells (Fig.3.19). The segregational stability of pWX11-bearing cells (Fig.3.22), appears to be due to an elevated plasmid copy number (Fig.3.23), and inactivation of tetracycline resistance, therefore removing a conferred selective disadvantage (Lee & Edlin, 1985), rather than the specific presence of the pDS1109-derived HaeIIA fragment. This leads to the assumption that ColE1 does not encode a par function, a conclusion in agreement with that arrived at by Kasner & Rownd, (1985).

3.7.3 Postulated pressure for the selection of plasmid-free host mutant cells

During transition to phosphate limitation, the copy number of pNT3 decreased from 170 to about 100 over a period of 40 generations (Fig.3.23). A 100:1 ratio of ColE1-type plasmid to host origins of replication, may represent an intracellular pressure that selects for



DNA sequence comparison of the par region of plasmid pSC101, with that of the region surrounding the unique EcoRI site of plasmid ColE1. The cruciform structures, differ in the number of base pairs that may be located within the stems, 13 and 7 for ColE1 and pSC101 respectively. The loops of each cruciform, however, do contain the homologous sequence CATT. Other sequence homology existing between the two regions, is indicated by the boxed nucleotides. The sequence ATAGNA, is present in both ColE1 and pSC101 as close direct repeats. *= homologous nucleotide, N= variable nucleotide.

host mutants capable of producing plasmid-free segregants. During chemostat culture of pWX11-bearing cells, the appearance of host mutant plasmid-free segregants coincided with a progressive increase in plasmid copy number (Fig.3.22 & 3.23). These host mutants exhibit alterations in their cell division cycle (see Chapter IV). The observed increases in plasmid copy number, therefore, may actually reflect alterations in the ratio of cell volume to chromosomal DNA content, suggesting that such mutants may possess defects in either the initiation, or rate of chromosome DNA replication, or both. Hence, for pNT3 and pWX11, the increases in plasmid copy number may mark the appearance of host mutants within the chemostat populations.

3.7.4 Stability functions derived from plasmids pSC101 and R1, and their effect on ColE1-type plasmid stability

Plasmids pWX9 and pOU93 exhibited markedly different copy numbers during chemostat culture (Fig.3.13). This may reflect the influence that each respective stability function may have, on the copy number of ColE1-type plasmids during chemostat culture.

To account for plasmid inheritance mediated by the par locus of pSC101, two opposing models have been postulated. The first of these, the 'single site inheritance' model of Hashimoto-Gotoh & Ishii (1982), proposes that it is the plasmid DNA molecules undergoing DNA replication, at a replicon-specific membrane site,

which are actively partitioned to daughter cells, while plasmid molecules residing in the cytoplasm are randomly distributed. In contrast, Tucker *et al.* (1984), following studies on the structure and function of the par region of pSC101, have proposed that it is the non-replicating plasmids that are actively partitioned, by a mechanism which relies on the ability of the par function to count individual molecules and evenly distribute them to daughter cells at division. Plasmids undergoing DNA replication were considered to be randomly distributed.

This latter model would imply that for each pWX9-bearing cell, plasmid copy number may be expected to be about equal to the average copy number for the population, and that copy number distribution within such a population should be very narrow. For plasmids that are randomly segregated, a wider distribution of plasmid copy numbers would be expected within a population of plasmid-bearing cells. If as postulated, a 100:1 ratio of ColE1-type to chromosomal replication origins represents an intracellular selective pressure, then this pressure will be evenly distributed within a population if the plasmid is likewise evenly distributed at cell division. Alternatively, the random segregation of plasmid replicons at cell division will lead to an uneven distribution of plasmid copies, resulting in only a certain proportion of the population experiencing the selective pressure, depending on how rapidly copy number is restored.

It may be assumed, therefore, that a postulated even

distribution of selective pressure during chemostat culture of pWX9-bearing cells, would be expected to lead to the earlier appearance of host mutants than would occur during the chemostat culture of pNT3- or pWX11-bearing cells; plasmids which may be randomly distributed. Alternatively, the par sequence derived from plasmid pSC101 may specify either a site, or function, that markedly increases intracellular pressure for the generation of host mutants. However, the appearance of postulated host mutants does seem to occur earlier during the chemostat culture of pNT3-bearing cells, than during the culture of pWX11-bearing cells, presumably reflecting an initial higher copy number for pNT3. Hence, the progressive increase in the copy number of pWX9 during chemostat culture, may reflect the presence of a substantial proportion of host mutant cells at the beginning of the culture. It is curious, that in each case of plasmid copy number increase, the copy number increases by about two per genome equivalent per generation, until the limit for plasmid DNA content is reached. This constant increase in plasmid copy number, may presumably reflect a total population increase in the ratio of cell volume to chromosomal DNA content occurring with each generation.

In contrast to pWX9, pOU93 did not exhibit any significant fluctuation in plasmid copy number during chemostat culture. A reduction in copy number, similar to that exhibited by pDS1109, may have been expected during the chemostat culture of pOU93-bearing cells, since this pBR322 derivative constitutively expresses

tetracycline resistance. Presence of the parB stability function, derived from plasmid R1, appears to counteract any selective disadvantage due to random plasmid segregation. Either the parB function mediates an even distribution of plasmid copies at cell division, as postulated for the par function of pSC101 (Tucker et al., 1984), or confers a selective advantage. Recent evidence suggests that parB may couple the host cell cycle to plasmid replication (Gerdes et al., 1985b), and may therefore function in a manner analogous to the ccd region of F (Ogura & Hiraga, 1983). This region in F encodes two polypeptide products, LetA (CcdA) and LetD (CcdB). LetD is postulated to function as a cell division inhibitor, whilst LetA suppresses the activity of the LetD polypeptide. Expression of LetA and LetD is thought to be linked to plasmid replication. If it is assumed that parB does indeed function via a mechanism similar to that postulated for ccd, and copies of the cell division inhibitor molecule are evenly distributed at cell division, then a counter selection to the selective disadvantage conferred by random plasmid segregation and the expression of tetracycline resistance will be present. Those cells inheriting a copy number greater than the population mean, will be unable to divide until their cell volume has increased sufficiently to allow a specific number of plasmid replications to be initiated, equal to the number of division inhibitor molecules inherited, so that these molecules can be inactivated. In contrast, those cells inheriting a copy number less than the population mean,

will have a deficiency in the number of plasmid replication origins available for plasmid replication, but will be able to initiate plasmid replication earlier. This should result in a period of division inhibition, consistent with the cell division cycle of those cells bearing the mean plasmid copy number. Cells that inherit a copy number equivalent to, or below the population mean, may therefore have a constant division period as a consequence of a balance between growth-rate (affected by tetracycline resistance expression), the rate of division inhibitor neutralization, cell volume and the rate of initiation of plasmid replication.

Recent studies on the ccd function of the F plasmid, have suggested that plasmid-free cells which arise, as a consequence of defective partitioning, are nonviable, forming filaments in which DNA synthesis is either reduced or nondetectable (Jaffe et al., 1985). If parB functions in a similar manner to the ccd function, then plasmid-free cells arising during chemostat culture of pOU93-bearing cells should be nonviable, this is obviously not the case. However, one possible explanation for the generation of viable pOU93-free segregants, is that the parB function may be unable to mediate such effects on either DNA synthesis, or the host cell division cycle, as a consequence of the generation of host mutant plasmid-free cells. Indeed, a slight increase in the copy number of pOU93 prior to the detection of plasmid-free segregants, suggests that host mutants defective in cell division may have been generated (Fig.3.13). Such postulated host mutants

arising within the chemostat population of pOU93-bearing cells, may be similar or identical to plasmid-free host mutants isolated during the chemostat culture of pWX9- and pWX11-bearing cells. It is notable that pOU93-free segregants did not exhibit kinetics of appearance analogous to those of pBR322-, pWX9- or pWX11-free cells, however, this may reflect the selective disadvantage conferred by expression of tetracycline resistance on pOU93-bearing cells, in comparison to the competitiveness of postulated plasmid-free host mutants.

3.7.5 Auxotrophic complementation and plasmid ColEl-type stability

Alternatives, to the employment of plasmid-encoded stability functions in ensuring the stable maintenance of ColEl-type cloning vectors, have made use of strategies which lead either to the 'suicide' of a host cell as a result of plasmid loss, or auxotrophic complementation, where a cloned gene indispensable for cell growth is encoded by the plasmid vector. The former strategy is exemplified by the approach of Rosteck & Hershberger (1983), who have constructed a plasmid vector expressing the cIts857 phage lambda repressor. A host strain bearing this recombinant plasmid was lysogenized by a repressor-defective lambda phage. Loss of the recombinant plasmid results in concomitant loss of the lambda repressor, leading to induction of the resident prophage and cell death. In contrast, Skogman & Nilsson (1984), who had previously obtained only a partial stabilization of a

tryptophan-operon-bearing pBR322 recombinant plasmid by use of the par locus of pSC101, subsequently adopted a strategy whereby the valS gene, encoding valyl t-RNA synthetase, was cloned into the unstable recombinant plasmid and shown to confer stable plasmid retention, for over 200 generations, in a thermosensitive valS host mutant of E.coli K-12 at the nonpermissive temperature.

The ensured stability of a plasmid-bearing population, by means which effectively force cells to retain a plasmid, would appear to require a greater input into the design and genetic manipulation of both plasmid and host strain, than encountered when simple plasmid-encoded stability functions are employed. Attempts to stabilize plasmid pWX15 by auxotrophic complementation of a glutamate-dependent strain of E.coli, highlights the importance of the genetic stability of both recombinant plasmid and host background to the stable retention of such recombinant plasmids. The presence of mobile genetic elements are obvious candidates able to promote DNA rearrangements, as suggested by postulated transposition of Tn10 into plasmid pWX15. Alternatively, cloned fragments derived from a bacterial chromosome may contain, in addition to desired sequences, other sequences also capable of mediating DNA structural rearrangements, a possible consequence of the presence of repeated sequences. In addition, sequence homology between cloned fragments and the host genome, particularly with regard to sequences encoding complementary functions, may also lead to

undesired sequence rearrangements, and a possible reversion to prototrophy. Therefore, it is essential that the mutational basis for auxotrophy be derived from an absence of sequences, within the host genome, encoding the function to be complemented. This should ensure minimal interaction between the recombinant plasmid and genomic sequences, in addition to minimizing reversion to prototrophy.

Finally, one area for concern may be the extent to which the copy number of a complementary auxotrophic plasmid is affected by either the cloned sequence, or the culture conditions. A sufficiently high plasmid copy number, as suggested by results obtained from the chemostat culture of cells bearing plasmids pWX9 or pWX11, may exact intracellular pressure for the selection of host mutants defective in cell division. Indeed, the appearance of characteristic 'pie crust' colonies, during ammonia-limited chemostat culture of strain WX100 bearing pWX15 (Fig.3.7), suggests that host mutants similar to those isolated during the chemostat culture of pWX9- and pWX11-bearing cells, may have likewise been generated.

3.7.6. Conclusions

Several factors are concluded to affect the segregational stability of ColE1-type plasmids. Among these are the recombinational proficiency of the host bacterium, which may promote plasmid multimerization, the genetic stability of both plasmid and host strain, particularly with respect to sequences that mediate DNA

rearrangements, plasmid copy number and whether a plasmid confers a reproductive advantage or disadvantage on its host cell.

In addition, it is postulated that plasmid ColE1 does not encode a par function, but does specify a function(s) conferring a selective disadvantage. Furthermore, it is proposed that a sufficiently high plasmid ColE1-type copy number, may represent an intracellular pressure for the selection of host mutants defective in both cell division and plasmid segregation. Cell division inhibition, associated with perturbations in chromosome replication, may be expected to increase the ratio of cell volume to chromosomal DNA content, possibly explaining increases in plasmid copy number occurring during chemostat culture of ColE1-type plasmid-bearing cells. Finally, the par function of plasmid pSC101, and the parB function of plasmid R1, may maintain a narrow plasmid copy number population mean distribution, as a consequence of mechanisms which ensure plasmid inheritance by the even distribution of a factor(s).

CHAPTER IV

ISOLATION AND PHENOTYPIC CHARACTERIZATION OF E. COLI K-12 MUTANTS DEFECTIVE IN PLASMID SEGREGATION

4.1 Introduction

The stable inheritance of a plasmid within a bacterial population, requires that at every division each daughter cell receives at least one copy of the plasmid replicon. In the case of unit-copy number plasmids such as F, there is now evidence to indicate that a plasmid-specified mechanism exists which ensures the presence within the host cell of at least two plasmid copies prior to cell division (Miki *et al.*, 1984a). For the intermediate-copy number plasmid pSC101, a *cis*-acting function designated *par* (Meacock & Cohen, 1980), can accomplish the active partition of plasmids via a possible outer membrane interaction (Gustafsson *et al.*, 1983).

In addition to plasmid-specified functions, there may also exist chromosome-encoded functions, distinct from those directly associated with plasmid replication, or inheritance, that may affect plasmid maintenance.

4.2 Mutant isolation, colony and cellular morphologies

4.2.1 Introduction

During the course of continuous culture studies, carried out to monitor the segregational stability of pBR322 derivatives carrying fragments derived from either pSC101 or pDS1109, plasmid-free mutant cells were isolated that when re-transformed, failed to efficiently maintain the pBR322 derivatives. These mutants appeared to be defective in both cell division and plasmid segregation.

4.2.2 Mutant isolation

E.coli K-12 strain W5445 carrying pWX11, has been shown to be stably maintained during phosphate-limited chemostat culture for at least 100 generations (Fig.3.22). Plasmid-free cells that arose beyond this period failed to predominate, persisting as an approximately 3% subpopulation for a further 150 generations. From this and a subsequent chemostat experiment, three plasmid-free strains were isolated. They were designated WX11-2, WX11-3 and WX11-8.

Plasmid pWX9 is also stably maintained in strain W5445, during phosphate-limited chemostat culture at both 37°C and 42°C, for at least 200 and 100 generations respectively (Fig.3.10). Plasmid-free cells that arose following 100 generations of chemostat culture at 42°C failed to predominate, persisting as an approximately 2% subpopulation. A strain isolated from this plasmid-free population was designated WX9-2.

4.2.3 Colony morphology

Strains WX9-2, WX11-2, WX11-3 and WX11-8 on L-agar at 37°C, are easily distinguishable from those of the parental strain W5445. The mutant strains give rise to a mixture of two colony types, the major colony type is large, circular and possesses a characteristic 'pie crust' outline, whereas the minor colony type is similar to that of the parental strain, which gives a smaller, circular, well defined colony, having a convex elevation. Further differences in colony morphology can be observed on A+B minimal agar at 30°C, when the mutant

strains give rise to mucoid colonies (Fig.4.1). Under these plating conditions strains WX11-2 and WX11-3 segregate mucoid and nonmucoid colonies. The efficiencies of plating onto rich medium, following overnight growth in minimal medium at 37°C, indicate that in comparison to the parent strain, strains WX11-2, WX11-3 and WX11-8, all exhibit slight reductions in their viabilities (Table 4.1). In contrast, strain WX9-2 plates with an efficiency slightly greater than that of the parent strain.

4.2.4 Cellular morphology

Three distinct cellular morphologies are exhibited by the mutant strains (Fig.4.2). The cells of strains WX9-2 and WX11-2 at 30°C, are similar in morphology to those of the parent strain (i.e., rod-shaped), however, some cells are either of slightly increased girth, or form nonseptate filaments of moderate length (Fig.4.2 A). Furthermore, a minority of the population exhibit a grossly bizarre morphology, in that the cells appear Y-shaped (Fig.4.2 B & C). The proportion of Y-shaped cells is enhanced by growth at 42°C, as is the proportion of cells forming filaments. In addition, the cells of strains WX9-2 and WX11-2 are also capable of exhibiting both filamentous, and Y-shaped morphologies in a single cell (Fig.4.2 D).

The cells of strain WX11-3 do not form filaments to any extent, although Y-shaped cells can be observed as a small proportion of the population. In addition, some cells exhibit an irregular pleomorphic shape, which is

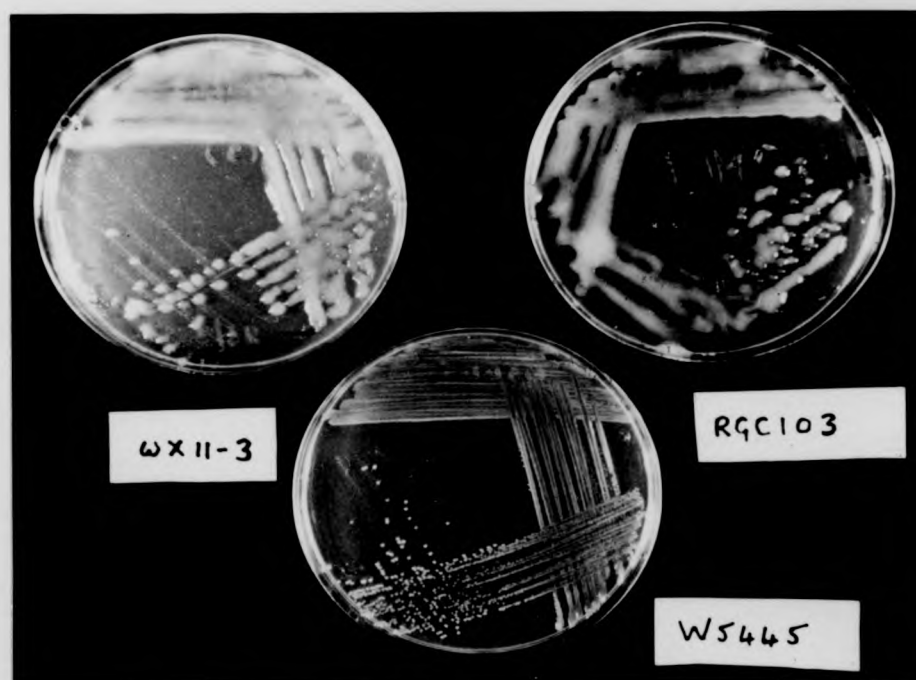


Figure 4.1

Nonmucoid colony phenotype of *E.coli* strain W5445, and mucoid colony phenotype of host mutant plasmid-free segregant WX11-3 and the capR9 (lon) strain RGC103, when grown on A+B minimal agar at 30°C.

<u>Strain</u> <u>designation</u>	<u>Plating efficiencies</u>	
	<u>minimal medium</u>	<u>rich medium</u>
W5445	1	0.99
WX9-2	1	1.14
WX11-2	1	0.79
WX11-3	1	0.89
WX11-8	1	0.72

Table 4.1

The efficiencies of plating onto rich medium of E.coli strain W5445 and mutant plasmid-free segregants of W5445, following overnight growth in A+B minimal medium at 37°C. Plating efficiencies were calculated as the average of two separate platings.

Figure 4.2

Scanning electron micrographs of the differing cellular morphologies exhibited by mutant plasmid-free segregant strains of *E.coli* W5445.

A) Filament formation in a cell of strain WX9-2 at 42°C. Magnification 10,000-fold.

B) Y-shaped formation in a cell of strain WX11-2 at 42°C. Magnification 20,000-fold.

C) a) Y-shaped formation in a cell of strain WX11-2 at 42°C. Magnification 40,000-fold.

b) Field of view observed following an angle of 45° rotation to the right of the line of view depicted in (a). Magnification 20,000-fold.

c) Field of view following an angle of 40° rotation to the right of the line of view depicted in (b). Magnification 20,000-fold.

d) Field of view following an angle of 40° rotation to the left of the line of view depicted in (a). Magnification 20,000-fold.

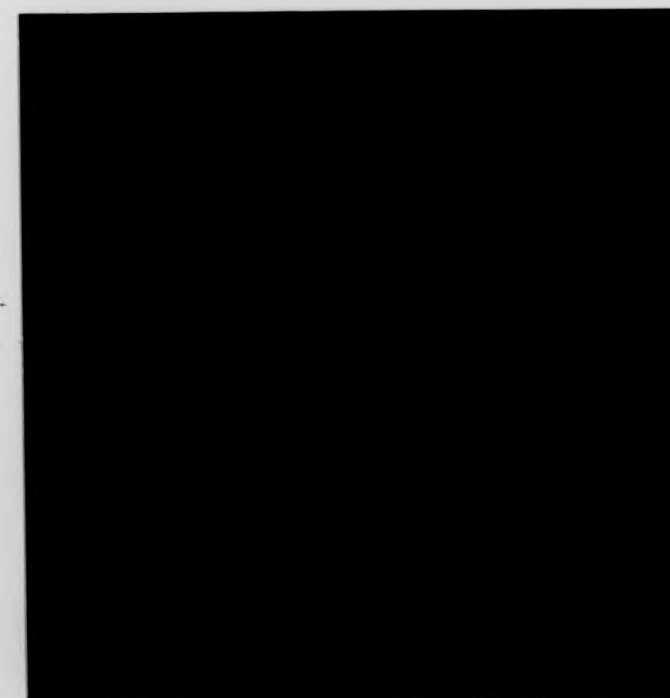
D) Filament and Y-shaped formation occurring in a cell of strain WX11-2 at 37°C. Lines of view depicted in (a) and (b), are separated by an angle of 180° rotation. Magnification 20,000-fold.

E) Minicell formation in a cell of strain WX11-8 at 42°C. Magnification 40,000-fold.

A)



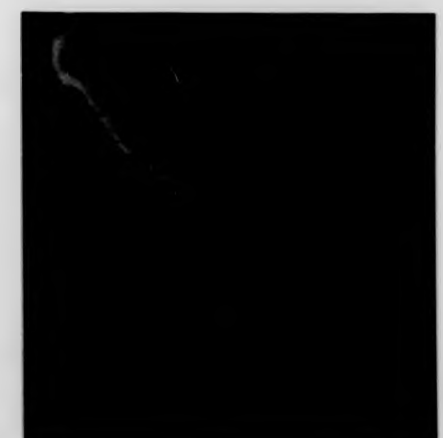
B)



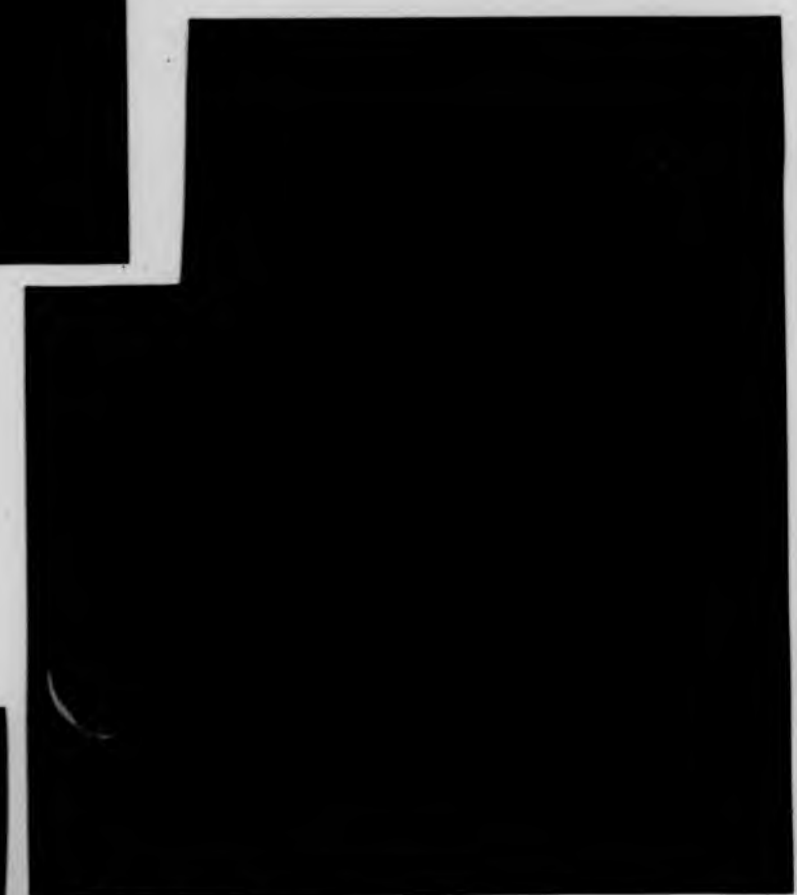
c)



c)



b)

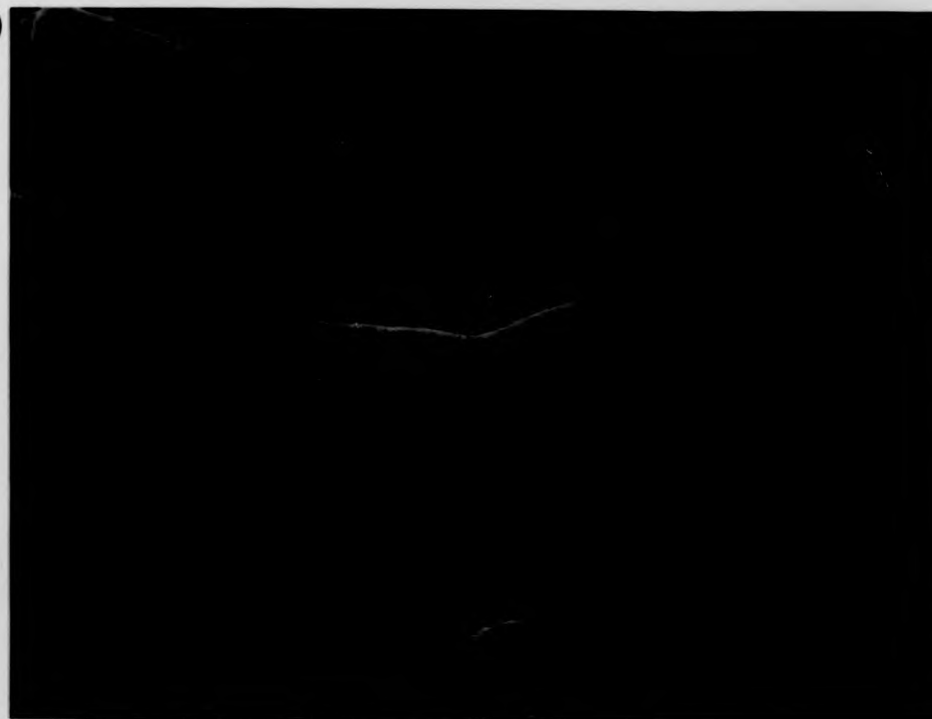


a)



d)

a)

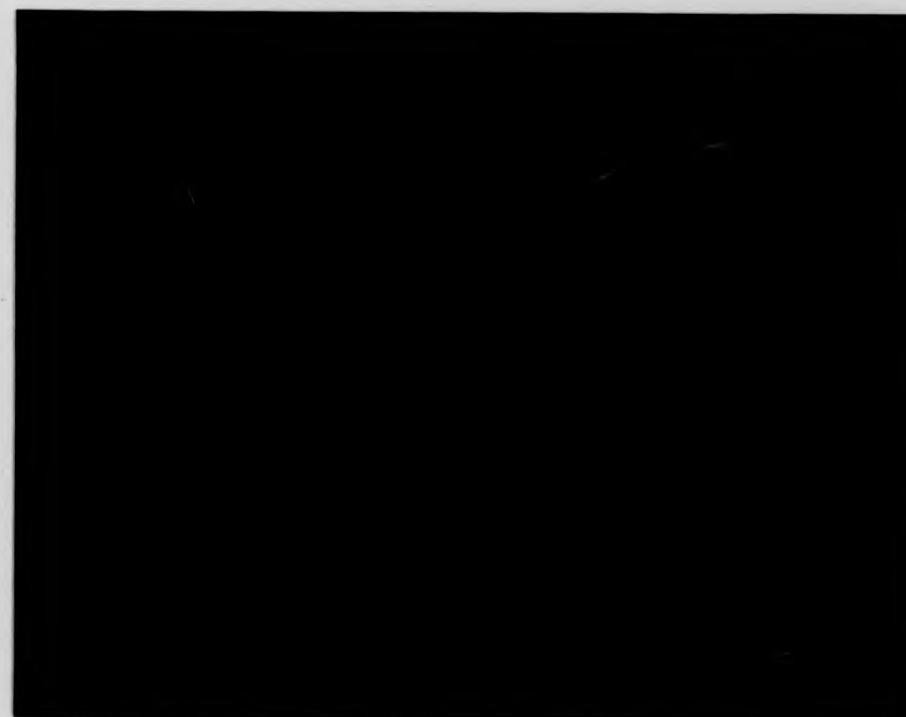


b)



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E)



enhanced by growth at 42°C.

In contrast to the above strains, cells of strain WX11-8 appear normal at 30°C, although a small proportion are slightly filamentous. However, following growth at 42°C, all cells form long filaments that produce at their ends, small, minicell like spheres (Fig.4.2 E).

4.2.5 Concluding remarks

The majority of host mutant plasmid-free segregants, isolated from chemostat cultures of pWX9- and pWX11-bearing cells, could not be readily maintained on rich medium. Host mutants WX11-2, WX11-3, WX11-8 and WX9-2 are isolates which survived subculture on rich medium, and therefore may represent a subclass of mutants arising within the original chemostat cultures. Indeed, plating efficiencies for WX11-2, WX11-3, WX11-8 and WX9-2 on minimal, in comparison to rich medium, would suggest that they may have been primarily selected as a consequence of their insensitivity to a nutritional shift. Hence, these mutants may only represent a small and selected proportion of potential host variants occurring within the chemostat populations.

Scanning electron microscopy indicates that a distinct cellular morphology is exhibited by strain WX11-8, in comparison to the other host mutants. Non-septate filament and minicell formation, suggests the occurrence of a defect in the positioning of the septal plane. Overproduction of the essential cell division gene product ftsZ (Ward & Lutkenhaus, 1985),

temperature-sensitive mutations in gyrB (Donachie *et al.*, 1984), or mutations in minB (Davie *et al.*, 1984), all lead to the production of minicells. However, overproduction of FtsZ does not induce minicell formation at the expense of normal septal formation, but appears to result in an increase in the number of septa formed. In contrast, gyrB^{ts} and minB mutations, lead to minicell formation at the expense of normal septum formation. Mutations in minB are presumed to represent defects only in the positioning of the septum, whilst gyrB^{ts} mutants are presumed to be defective in both chromosomal segregation and septal positioning. The pronounced temperature-sensitive effects on cellular morphology of strain WX11-8, suggests that this strain may have a temperature-sensitive defect in the gyrB subunit of DNA gyrase.

Cellular morphologies of WX11-2, WX11-3 and WX9-2, particularly with respect to Y-shaped cell formation, would suggest that it is the initial formation and positioning of the division septum which is defective, and may indicate either an interference with, or a defect in the functioning of the ftsZ gene product. Concentration of functional FtsZ polypeptide, during the *E. coli* cell cycle, is postulated to have a central role in the control of the division process (Holland & Jones, 1985). lon (Markovitz, 1977), tsM and certain ftsZ mutants (Belhumeur & Drapeau, 1984), all form filamentous cells and exhibit a mucoid colony phenotype. The lon, tsM and ftsZ mutations are presumed to interfere directly or indirectly with the functioning of

the essential cell division gene product ftsZ. Strains WX11-2, WX11-3 and WX9-2, may therefore, possess mutations allelic with those occurring in either lon, tsM or certain ftsZ strains, or some other mutation that indirectly interferes with the functioning of the ftsZ gene product.

4.3 U.V.-light resistance and antibiotic sensitivity

4.3.1 Introduction

The similarity in phenotypes of the four mutant strains with those of lon mutants (i.e., mucoidy and filamentation), prompted an investigation of their sensitivities to methyl methanesulphonate (MMS), the radiomimetic agent nitrofurantoin and U.V.-light irradiation, which are characteristic phenotypes of lon strains (Johnson, 1977; Kirby *et al.*, 1972; Berg *et al.*, 1976).

4.3.2 Results

Unlike their parent strain, the mutant plasmid-free segregants were sensitive to MMS at 42°C (Table 4.2 A), however, at 30°C and 37°C all strains were resistant. E.coli K-12 strains with mutations in the recA, recB, recC, polA or lon genes, are sensitive to nitrofurantoin (Jenkins & Bennett, 1976; Kirby *et al.*, 1972). All plasmid-free mutant strains, however, proved to be resistant to nitrofurantoin at 30°C, 37°C and 42°C (Table 4.2 A), while strains HB101 and RGC103, recA and capR9 (lon) respectively, were sensitive (Table 4.2 A).

A)

Strain designation:	Methyl methanesulphonate			Nitrofurantoin			Sodium azide			Relevant genotype
	30°C	37°C	42°C	30°C	37°C	42°C	30°C	37°C	42°C	
W5445	+	+	+	+	+	+	-	-	-	
WX9-2	+	+	-	+	+	+	-	-	-	
WX11-2	+	+	-	+	+	+	-	-	-	
WX11-3	+	+	-	+	+	+	-	-	-	
WX11-8	+	+	-	+	+	+	-	-	-	
HB101	NA	NA	NA	-	-	-	NA	NA	NA	recA
RGCL03	-	-	-	-	-	-	+	+	+	azi, cap89

B)

B)	<u>GyrA subunit</u>			<u>GyrB subunit</u>			<u>Relevant genotype</u>
	<u>Nalidixic acid</u>			<u>Novobiocin</u>			
<u>Strain designation:</u>	<u>Oxolinic acid</u>			<u>Coumermycin A₁</u>			
	30°C	37°C	42°C	30°C	37°C	42°C	
W5445	-	-	+	+	+	+	-
WX9-2	-	-	-	-	-	-	-
WX11-2	-	-	-	-	-	-	-
WX11-3	-	-	-	-	-	-	-
WX11-8	-	-	-	-	-	-	-
HB101	NA	NA	NA	NA	NA	NA	NA <u>recA</u>
RGCL03	-	-	-	-	-	-	- <u>azi, cap89</u>

Table 4.2

Sensitivity of the growth of *E. coli* strains on L-agar at 30°C, 37°C and 42°C, to the antimicrobial compounds, A) methyl methanesulphonate (250µl/litre), nitrofurantoin (4µg/ml), and sodium azide (100µg/ml), B) nalidixic acid (50µg/ml), oxolinic acid (0.5µg/ml), novobiocin (500µg/ml), and coumermycin A₁ (20µg/ml). +, = growth; -, = no growth; NA, = not applicable.

Furthermore, an analysis of the survival of strains following U.V.-light irradiation, indicated that all mutant plasmid-free segregants were more resistant to U.V.-light irradiation than their parent strain during exponential growth (Fig.4.3 A, a & b). However, similar U.V.-light sensitivities were exhibited by both the mutant and parent strains when grown to stationary phase (Fig.4.3 A, c to e).

Sensitivity of *lon* strains to U.V.-light irradiation can be suppressed chemically by post-irradiation incubation with the furan derivative DL-pantoyllactone (PL), (Kirby *et al.*, 1972; Nakayama *et al.*, 1982). The effects of this chemical on the U.V.-light sensitivities of the parent and mutant strains during exponential growth were determined (Fig.4.3 B). PL increased the U.V.-light resistance of the parent strain to a value comparable with that of untreated mutant strains (Fig.4.3 B, a). Strains WX11-2 and WX9-2 also exhibited increased U.V.-light resistance as a consequence of PL treatment (Fig.4.3 B, b & c), however, strain WX11-3 exhibited a decrease in its resistance to U.V.-light irradiation (Fig.4.3 B, d). On the other hand, PL treatment had no effect on the U.V.-light resistance of strain WX11-8 (Fig.4.3 B, e).

A decreased sensitivity to U.V.-light irradiation has been associated with mutations occurring in *gyrB* (von Wright & Bridges, 1981), but not *gyrA* (Chao & Tillman, 1982). It has been suggested that a mutational alteration of the DNA gyrase B subunit leads to an enhancement of the excision repair system, but to a

Figure 4.3

Sensitivity to U.V.-light irradiation of *E.coli* strain W5445 and mutant plasmid-free segregants of W5445. Results are presented as semi-logarithmic plots of the percentage colony forming units, surviving increasing periods of U.V.-light irradiation, measured in Joules m^{-2} .

A) Percentage survival U.V.-light irradiation following overnight incubation at 37°C on L-agar.

- a) Exponential phase cells of strains WX11-2; ▲—▲ , WX11-3; ■—■ , and W5445; ○—○ .
- b) Exponential phase cells of strains WX9-2; ▲—▲ , WX11-8; ■—■ , and W5445; ○—○ .
- c) Cells of strain W5445; ○—○ , exponential phase; ●—● , stationary phase.
- d) Stationary phase cells of strains WX11-2; ▲—▲ , WX11-3; ■—■ , and exponential phase cells of strain W5445; ○—○ .
- e) Stationary phase cells of strains WX9-2; ▲—▲ , WX11-8; ■—■ , and exponential phase cells of strain W5445; ○—○ .

A)

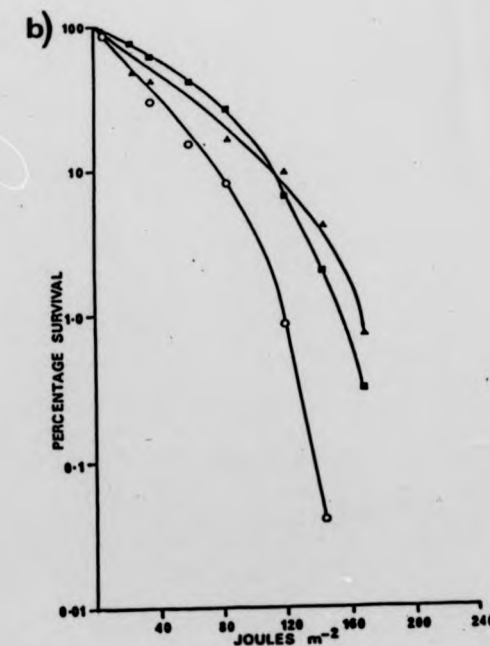
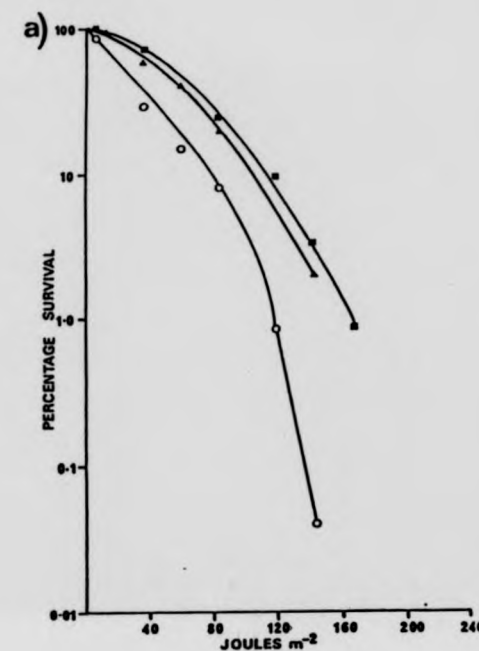
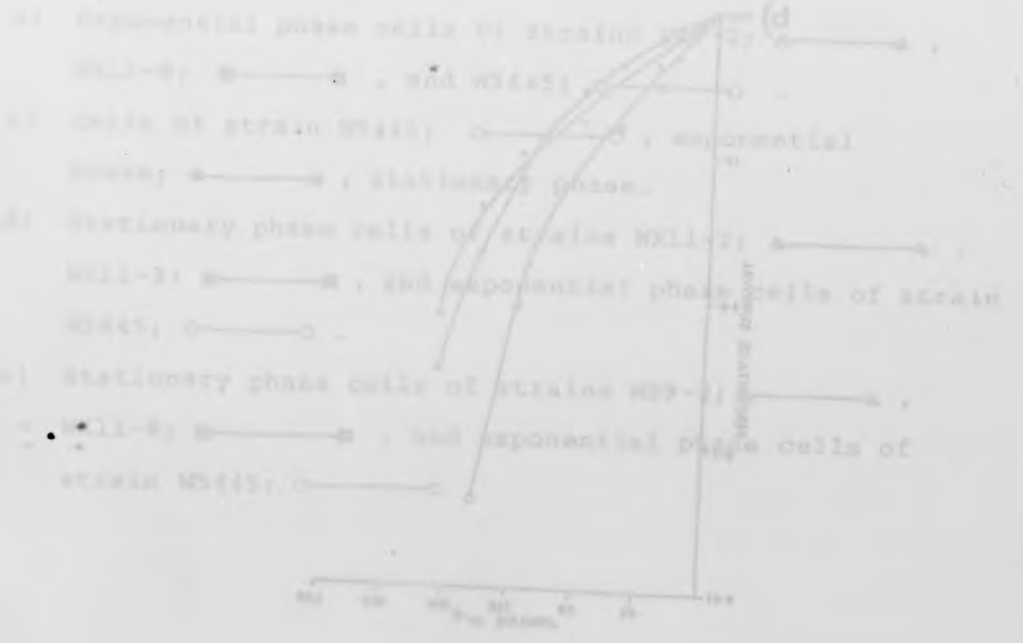


Figure 4.3

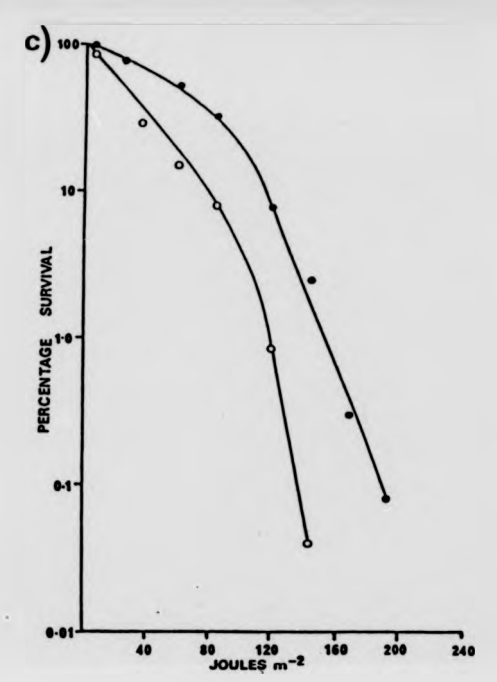
Sensitivity to U.V.-light irradiation of *Salmonella* strain W111 and mutant plasmid-free *Salmonella* strain W111. Results are presented as semi-logarithmic plots of the percentage surviving after 2 days of incubation following U.V.-light irradiation.

At 100% survival following U.V.-light irradiation at 17°C on 2 days.

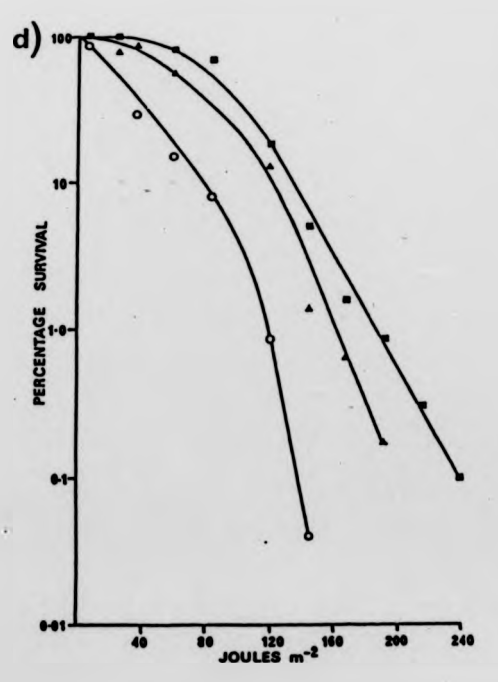
a) Exponential phase cells of strains W111-7; W111-3; and W111-2; b) Stationary phase cells of strains W111-7; W111-3; and W111-2; c) Stationary phase cells of strains W111-7; W111-3; and W111-2; d) Stationary phase cells of strains W111-7; W111-3; and W111-2; e) Stationary phase cells of strains W111-7; W111-3; and W111-2.



A)



d)



e)

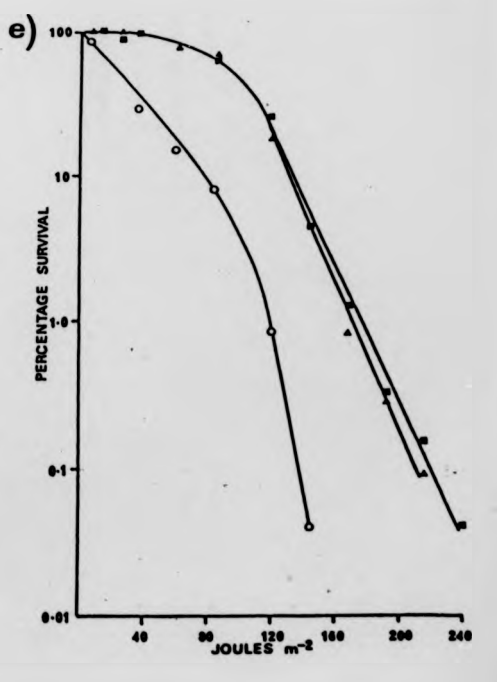
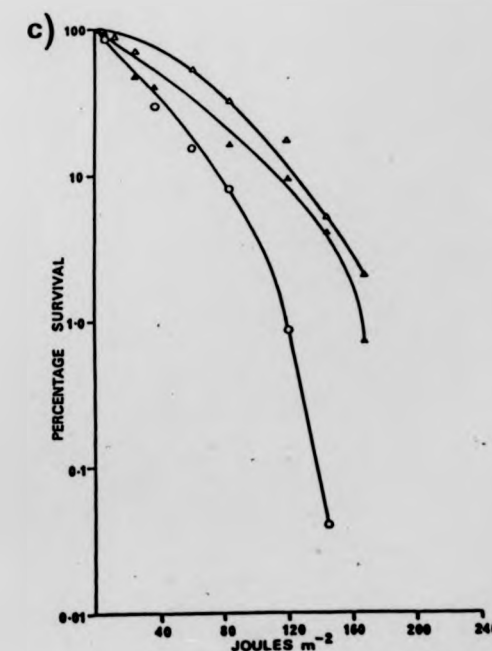
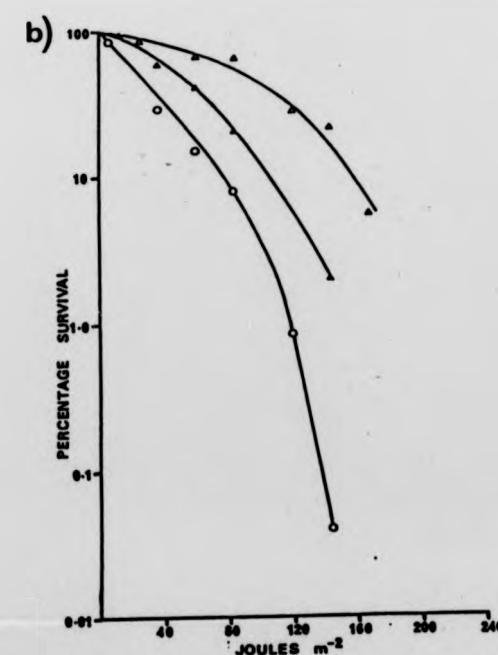
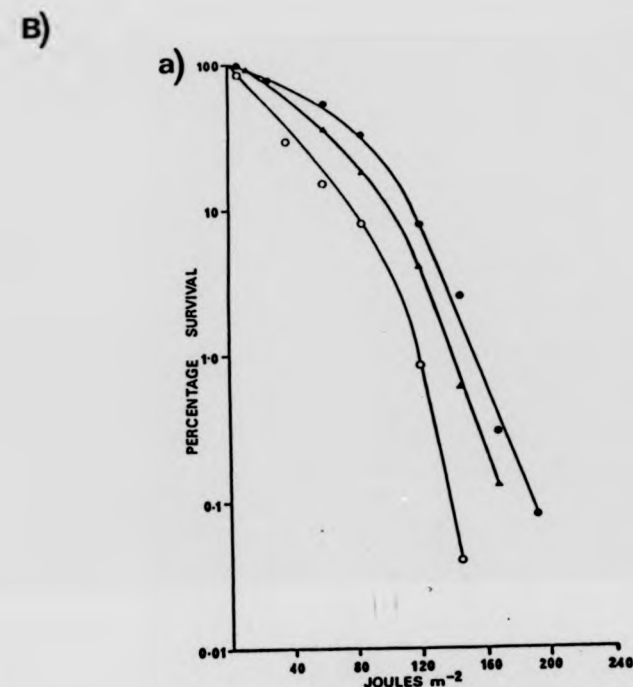


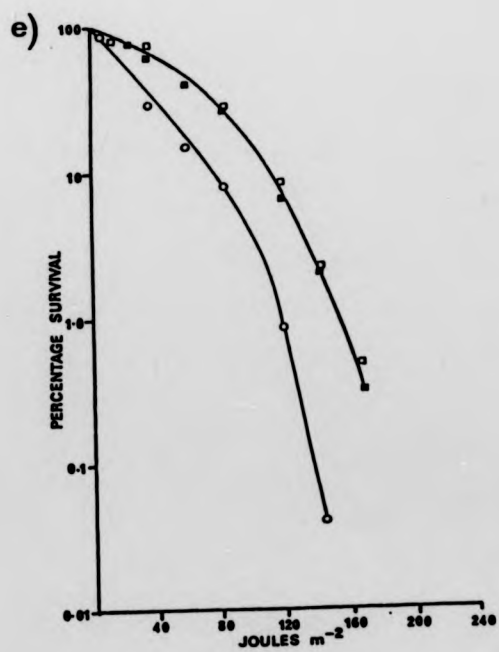
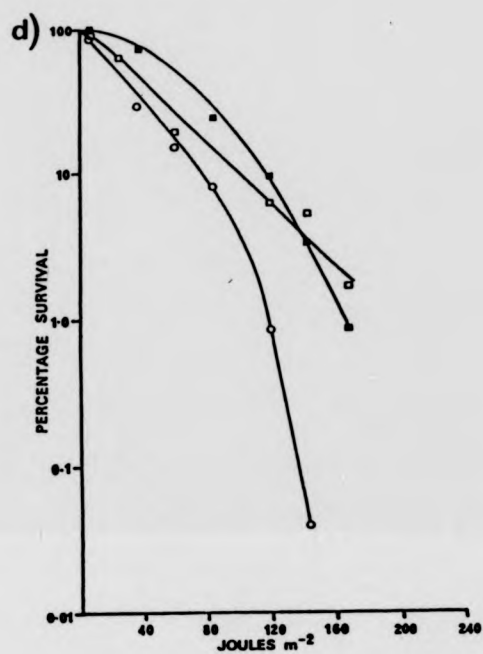
Figure 4.3 Con't.,

B) Percentage survival U.V.-light irradiation following overnight incubation at 37°C on L-agar and L-agar containing DL-pantooyllactone (13mg/ml).

- a) Cells of strain W5445 incubated on L-agar; \circ — \circ , exponential phase; \bullet — \bullet , stationary phase and L-agar containing DL-pantooyllactone; \blacktriangle — \blacktriangle , exponential phase.
- b) Exponential phase cells of strains W5445; \circ — \circ , and WX11-2; \blacktriangle — \blacktriangle , incubated on L-agar, and strain WX11-2; \triangle — \triangle , incubated on L-agar containing DL-pantooyllactone.
- c) Exponential phase cells of strains W5445; \circ — \circ , and WX9-2; \blacktriangle — \blacktriangle , incubated on L-agar, and strain WX9-2; \triangle — \triangle , incubated on L-agar containing DL-pantooyllactone.
- d) Exponential phase cells of strains W5445; \circ — \circ , and WX11-3; \blacksquare — \blacksquare , incubated on L-agar, and strain WX11-3; \square — \square , incubated on L-agar containing DL-pantooyllactone.
- e) Exponential phase cells of strains W5445; \circ — \circ , and WX11-8; \blacksquare — \blacksquare , incubated on L-agar, and strain WX11-8; \square — \square , incubated on L-agar containing DL-pantooyllactone.



B)



reduction in the efficiency of postreplication repair (von Wright & Bridges, 1981). The resistance of the mutant plasmid-free segregants to the gyrA inhibitors nalidixic acid and oxolinic acid, and the gyrB inhibitors novobiocin and coumermycin A₁, were determined. All strains were found to be sensitive to coumermycin A₁ at both 37°C and 42°C (Table 4.2 B). The mutants, like their parent strain, were found to be sensitive to nalidixic acid at both 30°C and 37°C, and to oxolinic at 37°C, however, the parent strain was found to be resistant to nalidixic acid at 42°C, whilst all strains were resistant to oxolinic acid at this temperature. Interestingly, filament formation exhibited by strain WX11-8 was found to be suppressed by the presence of oxolinic acid. In marked contrast to the other DNA gyrase inhibitors which were employed, the parent strain unlike the mutant strains, was found to be resistant to novobiocin at all temperatures tested (Table 4.2 B). An analysis of the sensitivities of the mutant strains to novobiocin at 37°C, indicated that sensitivity varied with novobiocin concentration (Table 4.3). Strain WX11-3 exhibited a tolerance to novobiocin at 300µg/ml, while strains WX9-2 and WX11-2 were tolerant to 100µg/ml, in contrast, strain WX11-8 was sensitive to 100µg/ml.

Finally, resistance to sodium azide, which is specified by a locus that maps within a region of the E.coli chromosome encoding other cell division loci, is associated with filament formation at 42°C (Yura & Wada, 1968). As a consequence, all strains were examined for

<u>Strain</u> <u>designation</u>	<u>Novobiocin concentration</u> <u>µg/ml</u>				
	500	400	300	200	100
W5445	+	+	+	+	+
WX9-2	-	-	-	-	(+)
WX11-2	-	-	-	-	(+)
WX11-3	-	-	(+)	(+)	+
WX11-8	-	-	-	-	-

Table 4.3

Sensitivity of the growth of E.coli strain W5445 and mutant plasmid-free segregants of W5445 on L-agar at 37°C, to varying concentrations of the antibiotic novobiocin. +, = growth; -, = no growth; (+), = sparse growth

their sensitivity to sodium azide at 37°C and 42°C, all strains proved to be sensitive (Table 4.2 A).

4.3.3 Concluding remarks

The phenotypes of mucoidy and filamentation have been associated with lon (Markovitz, 1977), tsM and certain ftsZ mutants (Belhumeur & Drapeau, 1984). Filament formation is also a phenotype associated with ruv (Attfield et al., 1985), and gyrB mutations (Orr et al., 1979), as well as mutations in a number of other distinct loci associated with chromosome replication, septum formation and the SOS response (Donachie et al., 1984).

The sensitivity of strains WX11-2, WX11-3, WX11-8 and WX9-2 to nitrofurantoin (Table 4.2 A), suggests that they are not defective in functions associated with DNA repair. Indeed, the four mutant strains showed greater resistance to U.V.-irradiation than their parent (Fig.4.3 A), which suggests that the mutation(s) they carry may not be allelic with lon, ruv or tsM mutations. lon strains, which carry a sul mutation, normally exhibit a U.V. resistance greater than that of the parent lon strain, but less than that of the wild-type parent strain (Fig.5.1 A,b, Chapter V). Since strains WX11-2, WX11-3, WX11-8 and WX9-2 all exhibit a U.V. resistance greater than that of the parent strain W5445, this would also suggest that the mutation(s) they carry are not allelic with lon sul mutations, as may have been suggested by their temperature-dependent sensitivity to MMS (Table 4.2 A & Table 5.2 A, Chapter V). The

similarities in U.V. resistance of exponentially growing cells of the mutant strains, with stationary phase cells of the parent W5445 (Fig.4.3 A), suggests a derepression of components of the SOS regulon to a level comparable with that of wild-type stationary phase cells (Karu & Belk, 1982). All four mutants, therefore, appear to exhibit a partial derepression of the SOS regulon.

The effects of the presence of PL in the post U.V. irradiation plating medium, appears to correlate with the cellular morphology of the mutant strains. Strains WX9-2 and WX11-2, which exhibit both Y-shaped and filamentous cell formation, showed increased rates of survival in the presence of PL, as did the parent strain W5445 (Fig.4.3 B, a to c). In contrast, strain WX11-3, which also forms Y-shaped cells but does not form filamentous cells to any great extent, showed a decreased rate of survival in the presence of PL (Fig.4.3 B, d). The presence of PL had no affect on the survival of the minicell producing strain WX11-8 (Fig.4.3 B, e). The mechanism by which PL mediates its effect on the survival of cells following U.V. irradiation is unknown. In the presence of PL, U.V.-induced lethal filament formation does not occur (presumably via the sfi-dependent pathway), and cells appear to divide normally (Kirby *et al.*, 1972). This would seem to be consistent with the increased rates of survival of strains W5445, WX9-2 and WX11-2 following U.V. irradiation, where suppression of the lethal effects of filament formation appear to result in a

decrease in U.V. sensitivity. This may suggest that the pathway of division inhibition in strains WX9-2 and sfi-dependent WX11-2 is able to respond to suppression by PL. On the other hand, strain WX11-3, which does not form filamentous cells to any great extent, and strain WX11-8, which forms minicells at the expense of normal septum formation, exhibit very different rates of survival in the presence of PL. The sfi-dependent pathway of strain WX11-3, may be defective and unable to respond to suppression by PL, leading to increased U.V. sensitivity. This strain may therefore possess two mutations, one leading to the formation of Y-shaped cells, as observed in strains WX9-2 and WX11-2, but the other suppressing filamentous cell formation. In the case of strain WX11-8, the presence of PL may only alter the rate at which nonviable minicells are produced, and therefore have no significant effect on the rate of survival of WX11-8 cells following U.V. irradiation.

The decrease in sensitivity to U.V. irradiation of the four mutant strains, appears to be analagous to that observed with gyrB mutants (von Wright & Bridges, 1981). In contrast to the parent strain W5445, all four mutant strains were sensitive to the gyrB inhibitor novobiocin at 500µg/ml (Table 4.2 B). Sensitivity to novobiocin varied with antibiotic concentration (Table 4.3), and appears to correlate with cellular morphology. Strain WX11-3 which does not readily form filamentous cells, but is capable of exhibiting a Y-shaped cellular morphology, tolerated a novobiocin concentration of 300µg/ml. Strains WX9-2 and WX11-2, which form

filamentous as well as Y-shaped cells, are tolerant to 100µg/ml, while the minicell producing strain WX11-8, is sensitive to 100µg/ml. These variations in sensitivity to differing novobiocin concentrations, may however, reflect changes in the cell envelopes of the mutant strains and therefore accessibility of the antibiotic. Indeed, all strains were sensitive to the gyrB inhibitor coumermycin A₁ (Table 4.2 B), an antibiotic not associated with problems of cell envelope exclusion. Likewise, the differential sensitivity of the parent and mutant strains to nalidixic acid at 42°C (Table 4.2 B), may reflect a similar situation. Hence, resistance of strain W5445 to nalidixic acid and novobiocin could be either the consequence of a mutation in the gyrA or gyrB subunit, specifically blocking the activity of these antibiotics, or simply the result of cell envelope exclusion. However, the sensitivity of E.coli strain AB1157 to nalidixic acid and novobiocin (Table 5.2 B, Chapter V), would seem to suggest that cell envelope exclusion may not be the basis for strain W5445's resistance to these antibiotics, but that some genuine difference occurs in the DNA gyrase of this strain. Indeed, the suppression by oxolinic acid of temperature sensitive cell filament formation by strain WX11-8, suggests that this strain may possess a defect in DNA gyrase. Likewise, strains WX9-2, WX11-2 and WX11-3 may also possess defects in DNA gyrase. Therefore, it is possible that an indirect partial induction of the SOS response, as a consequence of defects in DNA gyrase, may result in the induction of the division inhibitor Sula

and the RecF pathway of recombination (Drlica, 1984; Kolodner *et al.*, 1985), leading to interference with the functioning of FtsZ and enhanced plasmid recombination respectively, affecting both the pattern of cell division and plasmid segregation.

4.4 Effect of host mutant backgrounds on plasmid configuration

4.4.1 Introduction

The possible influence of the host mutant backgrounds on plasmid configuration were investigated. The four mutant strains were transformed with plasmids pBR322, pWX9, pWX11, pPM31, pOU93, pDS1109 and RSF2124 DNA. All strains were found to be transformable with each of the plasmids tested, suggesting that the mutant strains were not significantly defective in the replication of ColEI-type replicons.

4.4.2 Results

a) Plasmid multimerization

Rapid, small-scale plasmid isolation from transformed mutant strains and agarose gel analysis, indicated that the configuration of certain plasmid DNA species, compared with that found in the parent strain, could vary, and that this variation appeared to be due to the multimerization of plasmid DNA molecules (Fig. 4.4 & 4.5). Furthermore, the extent to which these plasmids multimerized appeared to vary with growth conditions, plasmid and host background (Table 4.4).

Figure 4.4

Agarose gel electrophoresis demonstrating the linear migration of multimeric forms of plasmid pWX9.

A) Gel electrophoresis of rapid, small-scale plasmid preparations obtained from *E.coli* K-12 strains bearing plasmid pWX9 cultured under ampicillin selective pressure at 37°C, lanes a and b) strain HW27 incubated in minimal medium and minimal medium containing DL-pantoyllactone respectively. This strain is a spontaneous derivative of AB1899, which is a *lon*-1 derivative of strain AB1157. Strain HW27 segregates out nonmucoid colonies (the strain was a generous gift from S.B. Primrose, G.D. Searle Limited, High Wycombe, U.K.), c and d) strain WX11-2 incubated in minimal medium, and minimal medium containing DL-pantoyllactone respectively, e and f) strain W5445 incubated in minimal medium and minimal medium containing DL-pantoyllactone respectively.

B) Semi-logarithmic plot of plasmid oligomers, in kilobase pairs of supercoiled plasmid DNA, verses their migrational distance in (A) above.

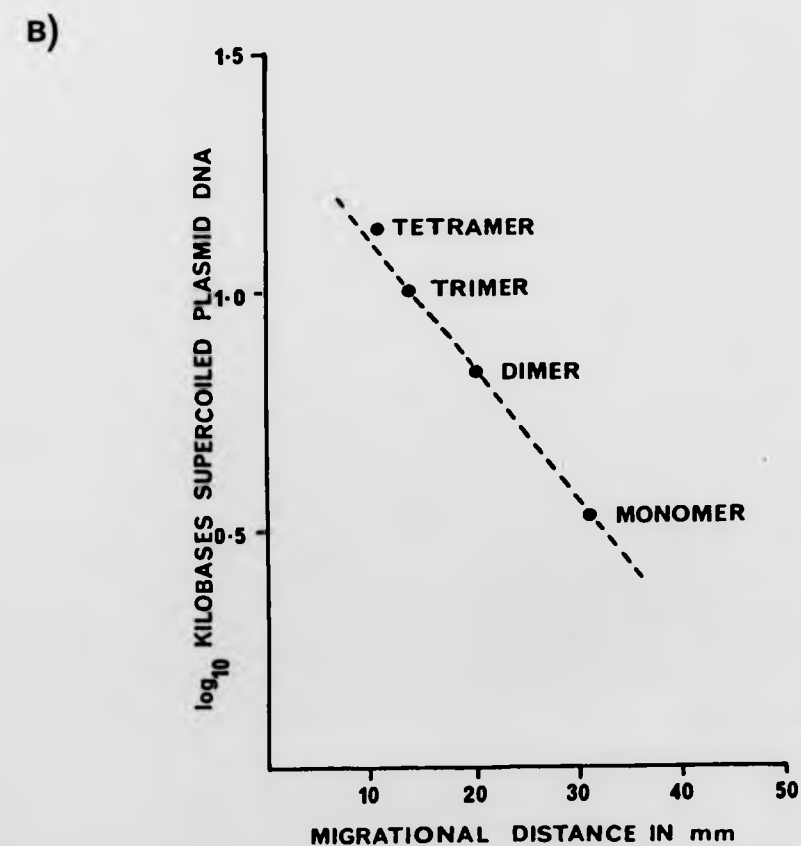
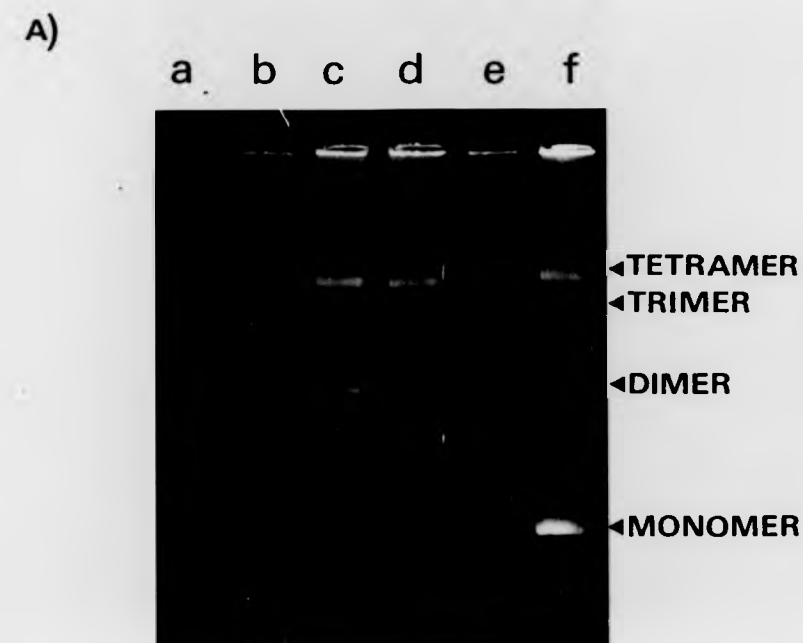


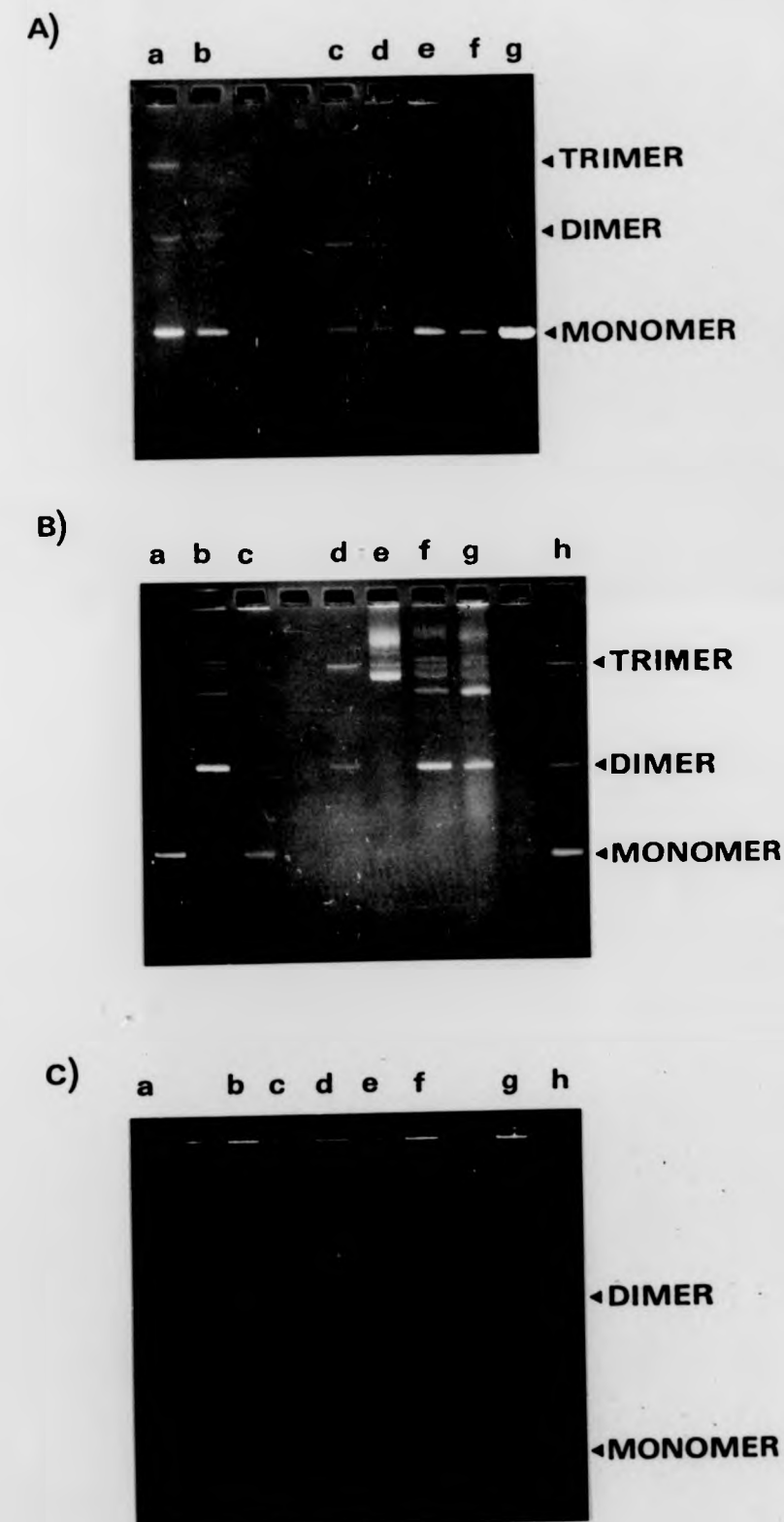
Figure 4.5

Representative agarose gel electrophoresis of multimeric plasmid DNA profiles, obtained from rapid, small-scale plasmid preparations of *E.coli* K-12 strains, cultured at 37°C under appropriate antibiotic selective pressure.

A) Plasmid pBR322 isolated from, lanes a and b) strain W5445 grown in rich and minimal medium respectively containing DL-pantoyllactone, c to f) strains WX11-8, WX11-3, WX11-2 and WX9-2 respectively, grown in minimal medium containing DL-pantoyllactone, g) purified plasmid pBR322 DNA isolated from strain W5445 following CsCl-EtBr density gradient centrifugation.

B) Plasmid pWX9 isolated from, lanes a, c and e to g) strains W5445, WX11-3, WX11-8, WX11-2 and WX9-2 respectively, grown in minimal medium containing DL-pantoyllactone, b and d) strains WX9-2 and WX11-2 respectively, grown in rich medium containing DL-pantoyllactone, h) purified plasmid pWX9 DNA isolated from strain W5445 following CsCl-EtBr density gradient centrifugation.

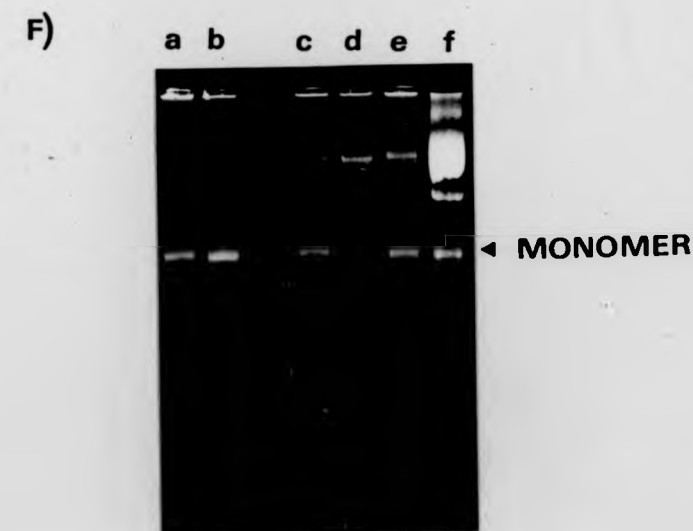
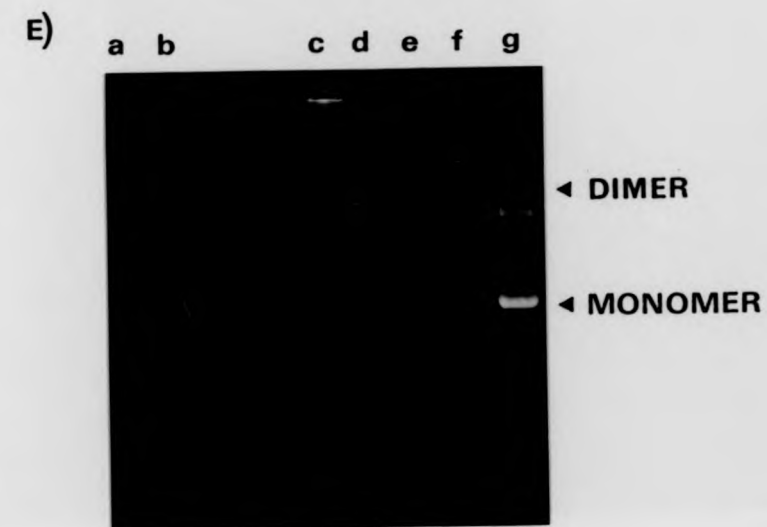
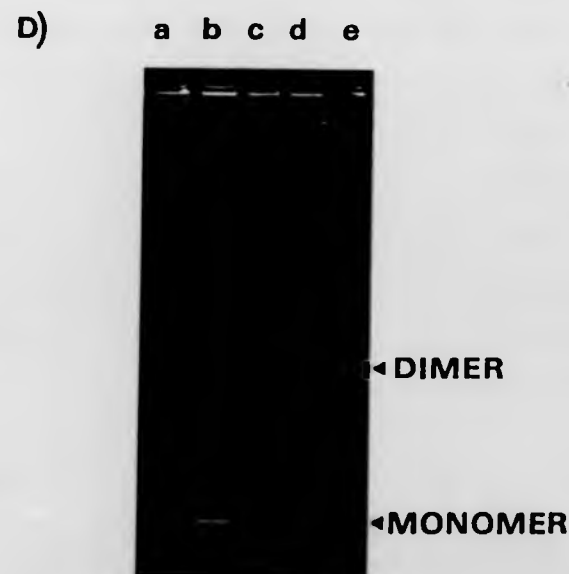
C) Plasmid pWX11 isolated from, lanes a, and d to f) strains WX9-2, WX11-8, WX11-3 and WX11-2 respectively, grown in minimal medium, b and c) strains X156 and RGC103 respectively grown in rich medium (see Chapter V), g) strain WX11-8 grown in rich medium, and h) purified plasmid pWX11 DNA isolated from strain W5445 following CsCl-EtBr density gradient centrifugation.



D) Plasmid pPM31 isolated from, lanes a to d) strains WX11-2, W5445, WX11-8 and WX11-3 respectively grown in minimal medium, e) purified plasmid pPM31 DNA isolated from strain W5445 following CsCl-EtBr density gradient centrifugation.

E) Plasmid pOU93 isolated from, lanes a and b) strains RGC103 and X156 respectively, grown in rich medium (see Chapter V), c to f) strains WX11-3, WX11-2, WX9-2 and W5445 respectively grown in rich medium, g) purified plasmid pOU93 DNA isolated from strain W5445 following CsCl-EtBr density gradient centrifugation.

F) Plasmid pDS1109 isolated from, lane a) strain WX11-8 grown in rich medium, c and e) strains WX11-8 and WX11-2 respectively grown in minimal medium f) purified plasmid pDS1109 DNA isolated from strain W5445 following CsCl-EtBr density gradient centrifugation, d) plasmid pWX11 isolated from strain W5445 grown in minimal medium, b) plasmid RSF2124 isolated from W5445 grown in minimal medium.



A) Minimal medium

Plasmid designation: pBR322 pWX9 pWX11 pRM31 pOU93 pDS1109 RSF2124

Strain designation:

W5445	M	M	M	M	M(D)	M	M
WX9-2	M	D	D	M	M	M	M
WX11-2	M	D	D	M	M	M	M
WX11-3	M	M	M	D	M	M	M
WX11-8	M	T	M	M	M	M	M

+ DL-pantoyllactone:

W5445	M	M	M				
WX9-2	M	D	D				
WX11-2	M	D	D				
WX11-3	M	M	M				
WX11-8	M	T	M				

B) Rich medium

Plasmid designation: pBR322 pWX9 pWX11 pRM31 pOU93 pDS1109 RSF2124

Strain designation:

W5445	M	M(D)	M	M	M	M	M
WX9-2	M	D	D	M	M	M	M
WX11-2	M	D	D	M	M	M	M
WX11-3	M	M	D	D	M	M	M
WX11-8	M	D	D	M	M	M	M

+ DL-pantoyllactone:

W5445	M	M	M				
WX9-2	M	D	D				
WX11-2	M	D	D				
WX11-3	M	M	D				
WX11-8	M	T	D				

Table 4.4

Configuration of the lowest, clearly observable plasmid multimer for plasmids pBR322, pWX9, pWX11, pRM31, pOU93, pDS1109 and RSF2124, as determined by migrational analysis in agarose gels following rapid, small-scale plasmid isolation from transformants of *E. coli* strain W5445 and mutant plasmid-free segregants of W5445. Transformants were cultured under ampicillin selective pressure and incubated at 37°C in either A) minimal or B) rich medium, with or without the presence of DL-pantoyllactone; M = monomer; D = dimer; (D) = dimer in addition to monomer also clearly observable within agarose gel; T = trimer.

When grown in minimal medium, all plasmids isolated from the parent strain W5445 were found to exist mainly as monomers (Table 4.4 A). In contrast, pWX9 and pWX11 (Fig.4.4 A, lane c & Fig.4.5 C, lanes a & f), were found to exist mainly as dimers in strains WX9-2 and WX11-2, while all other plasmids in these strains existed mainly as monomers. In strain WX11-3, pPM31 existed mainly as a dimer (Fig.4.5 D, lane d), and in strain WX11-8, pWX9 existed mainly as a trimer, all other plasmids in these strains migrated mainly as monomers.

Following growth of plasmid-bearing mutant strains in rich medium, the only variations found to the above patterns (Table 4.4 B), were that pWX11 existed mainly as a dimer in strain WX11-3 (Fig.5.2 D, lane e), as did both pWX9 and pWX11 in strain WX11-8. Furthermore, when the parent and mutant strains carrying pWX9 were incubated in rich medium at 25°C, 37°C and 42°C (Table 4.5), only strain WX11-8 showed a variation in plasmid configuration with growth temperature. At 25°C, pWX9 existed mainly as a monomer in strain WX11-8, while at 37°C and 42°C, the plasmid existed mainly as a dimer.

The effects of incubation with PL on the configuration of plasmids pBR322, pWX9 and pWX11, were investigated in both minimal and rich medium at 37°C (Table 4.4). The presence of PL affected the configuration only of plasmid pWX9 in strain WX11-8, and only when cultured in rich medium. Plasmid pWX9 existed as a trimer in the presence of PL, in contrast to existing as a dimer when cultured in rich medium only.

<u>Strain</u> <u>designation</u>	<u>Temperature</u>		
	25°C	37°C	42°C
W5445	M	M	M
WX9-2	D	D	D
WX11-2	D	D	D
WX11-3	M	M	M
WX11-8	M	D	D

Table 4.5

Configuration of the lowest, clearly observable plasmid multimer for plasmid pWX9, as determined by migrational analysis in agarose gels following rapid, small-scale plasmid isolation, from transformants of *E.coli* strain W5445 and mutant plasmid-free segregants of W5445, cultured under ampicillin selective pressure and incubated at either 25°C, 37°C or 42°C in rich medium. M = monomer; D = dimer.

Finally, it must be noted that plasmids isolated from strains WX9-2 and WX11-3 appeared to exhibit very slight differences in migration, following agarose gel electrophoresis, when compared with plasmids isolated from strains W5445, WX11-2 and WX11-8 (Fig.4.5 C, lane e Fig.4.7 A, lane b & Fig.4.7 B, lanes e & h).

b) Characterization of plasmid dimers

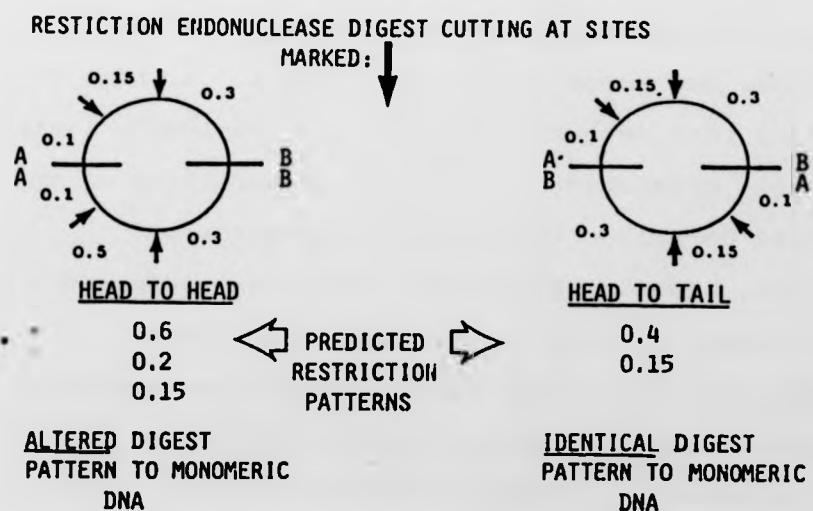
Following chloramphenicol amplification, plasmids pWX9 and pWX11 were isolated by CsCl-EtBr density gradient centrifugation, from strain WX11-8 grown at 37°C in rich medium (Fig.4.6 A). DNA preparations of dimeric plasmids pWX9 or pWX11 were designated pWX9-8 and pWX11-8 respectively, and were used to transform strain W5445 and mutant plasmid-free segregants of W5445. Analysis on agarose gels of rapid, small-scale plasmid preparations obtained from resultant transformants, indicated that following transformation into the parent strain W5445, resolution of the plasmid dimers does not occur (Table 4.6 & Fig.4.7 B, lane d), suggesting that such multimers may be covalently linked rather than catenated, the latter a possibility of the postulated defect(s) in DNA gyrase (Kreuzer & Cozzarelli, 1980). In contrast, both monomeric and dimeric forms of plasmid pWX11 (pWX11-8), were detectable in rapid, small-scale plasmid preparations from transformants of strains WX11-3 and WX11-8, following growth in minimal medium (Fig.4.7 B, lanes g & h), however, plasmid DNA bands corresponding to monomeric forms of pWX11 (pWX11-8), appeared to be less

Figure 4.6

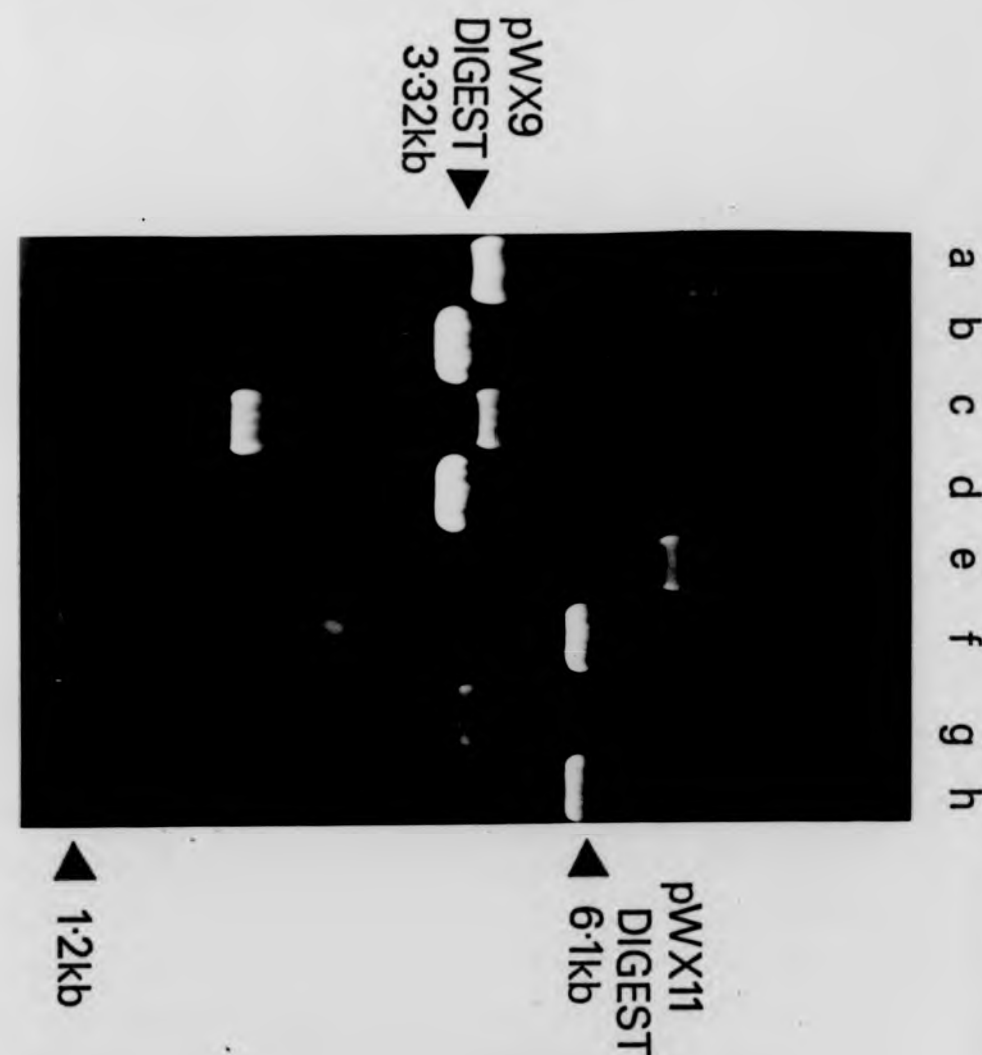
A) Comparative EcoRI restriction endonuclease analysis of purified monomeric and dimeric plasmid DNA preparations harvested from CsCl-EtBr density gradients. Lanes a and b) undigested and digested pWX9-8 DNA isolated from strain WX11-8, c and d) undigested and digested pWX9 DNA isolated from strain W5445, e and f) undigested and digested pWX11-8 DNA isolated from strain WX11-8, g and h) undigested and digested pWX11 DNA isolated from strain W5445.

B) Schematic representation of head-to-head or head-to-tail, covalently closed circular dimeric forms of plasmid DNA. Restriction endonuclease digest patterns allowing estimation of the multimeric configuration of plasmid DNA, are indicated.

B)



A)



Plasmid designation

Strain designation	minimal		rich	
	medium		medium	
	pWX9-8	pWX11-8	pWX9-8	pWX11-8
W5445	D	D	D	D
WX9-2	D	D	D	D
WX11-2	D	D	D	D
WX11-3	D	D(M)	D	D
WX11-8	D	D(M)	D	D
+ DL-pantoyllactone				
W5445	D	D	D	D
WX9-2	D	D	D	D
WX11-2	D	D	D	D
WX11-3	D	D	D	D
WX11-8	D	D	D	D

Table 4.6

Configuration of the lowest, clearly observable plasmid multimer for plasmids pWX9-8 and pWX11-8, as determined by migrational analysis in agarose gels following rapid, small-scale plasmid isolation from transformants of *E.coli* strain W5445, and mutant plasmid-free segregants of W5445. Transformants were incubated at 37°C under ampicillin selective pressure in either minimal, or rich medium, with or without the presence of DL-pantoyllactone; D = dimer; (M) = monomer in addition to dimer also observable within agarose gel.

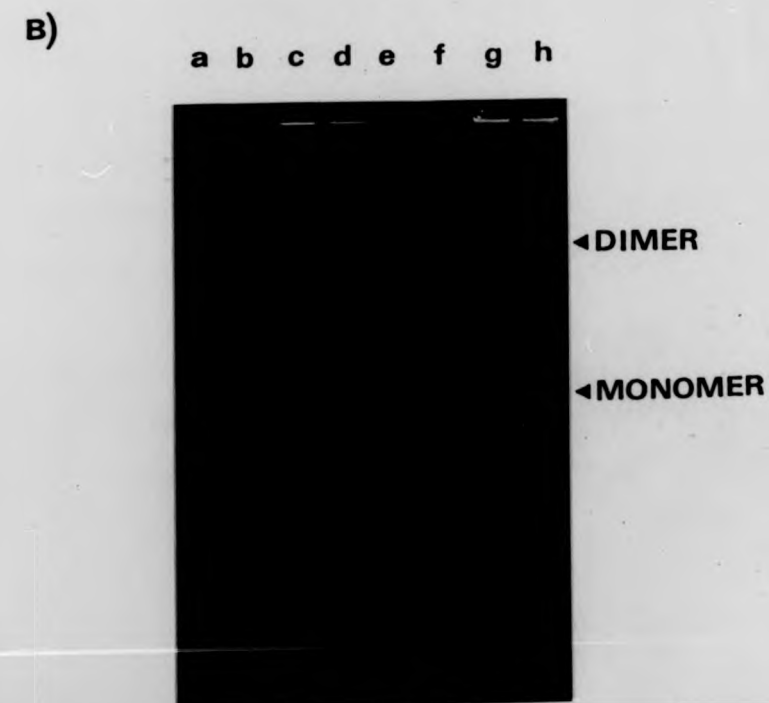
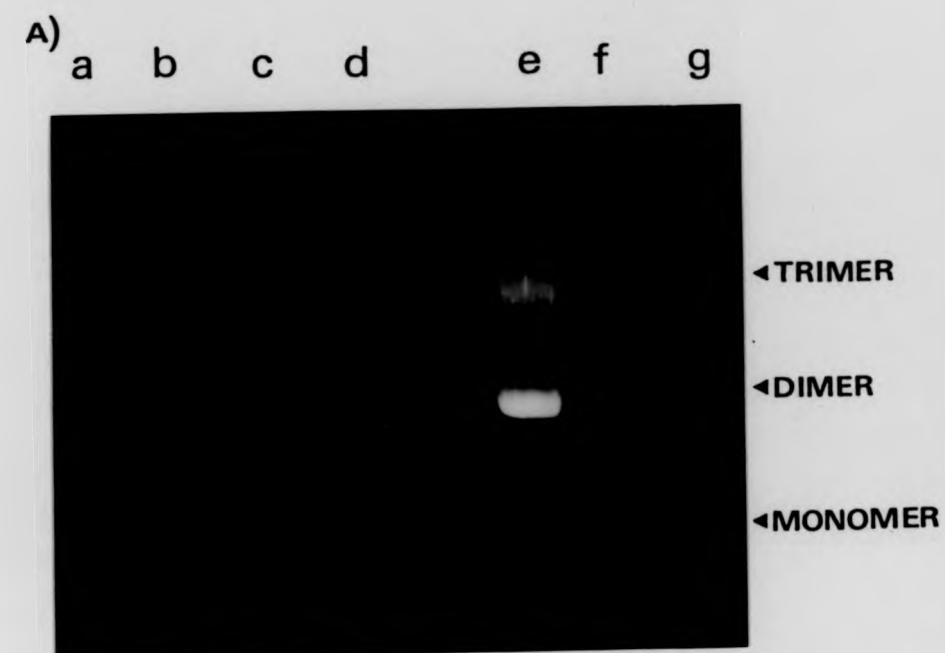


Figure 4.7

Representative agarose gel electrophoresis of multimeric plasmid DNA profiles, obtained from rapid, small-scale plasmid preparations of *E.coli* K-12 strains, cultured at 37°C under ampicillin selective pressure.

A) Plasmid pWX9-8 isolated from, lanes a to d) strains WX11-8, WX11-3, WX11-2 and WX9-2 respectively, grown in minimal medium containing DL-pantoyllactone, f) plasmid pWX9 isolated from strain PAM163 (see Chapter V), grown in minimal medium, e and g) purified plasmids pWX9-8 and pWX9 DNA isolated from strains WX11-8 and W5445 respectively, following CsCl-EtBr density gradient centrifugation.

B) Plasmid pWX11-8, lanes a and b) purified plasmids pWX11 and pWX11-8 DNA isolated from strains W5445 and WX11-8 respectively, following CsCl-EtBr density gradient centrifugation, c) plasmid pWX11-8 isolated from strain WX11-8 grown in rich medium, and from d to h) strains W5445, WX9-2, WX11-2, WX11-8 and WX11-3 respectively, grown in minimal medium.



intense than the DNA bands corresponding to the dimeric form. Furthermore, incubation of transformed strains with PL, failed to lead to any alteration in the multimeric configuration of pWX9-8 or pWX11-8 (Table 4.6 & Fig.4.7 A, lanes a to d), with the exception that monomeric forms of pWX11 (pWX11-8), could not readily be observed in plasmid DNA preparations from strains WX11-3 and WX11-8. In addition, it is puzzling to note that transformation of strain WX11-8 with pWX9-8, in contrast to pWX9, does not result in the isolation of mainly trimeric forms of pWX9 (Table 4.4 & 4.6).

To determine whether the plasmid multimers were covalently linked in a head-to-tail, or head-to-head manner, plasmid DNA was restricted with EcoRI and analysed by agarose gel electrophoresis (Fig.4.6 A). The restriction fragments produced by the dimers were of identical size to those produced by the respective plasmid monomers, suggesting that the dimers possess an head-to-tail configuration (Fig.4.6 B). In addition, endonuclease restriction of plasmid DNA confirmed that alterations in plasmid mobilities following gel electrophoresis, were not the consequence of linearization, but of multimerization.

4.4.3 Concluding remarks

Successful transformation with a number of different plasmids indicates that the four mutant strains are not significantly defective in plasmid replication, initiated at either pBR322, ColE1 or pl5A origins. The extent of plasmid multimerization, however, appears to

vary with growth conditions, plasmid and host mutant background. Plasmids pWX9 and pWX11, appear to be more prone to multimerization in the host mutants than the other plasmids examined. This does not appear to be attributable to pBR322-derived replication, since plasmids pBR322 and pOU93 did not readily multimerize. This suggests that both pWX9 and pWX11 may contain sites, presumably located within the respective fragment inserts, that mediate site-specific interplasmidic recombination. The terminal repeat module, which flanks the Tn3-derived sequence in pWX11, may function in this respect (Kleckner, 1981), as may the RS-beta sequence, within the par region of the pSC101-derived sequence, in pWX9 (Novick *et al.*, 1984). Plasmid pPM31, however, did not multimerize to the same extent as pWX9, which may suggest that some difference occurs in respect of the DNA substrate, or as a consequence of plasmid replication initiated from a pl5A origin. However, it was notable that multimerization of pPM31 occurred only in strain WX11-3. Plasmids isolated from strains WX9-2 and WX11-3 appeared to have slightly greater apparent molecular weights, as determined by gel electrophoresis. Either, these plasmids have increased in size as a consequence of the insertion of additional DNA, or strains WX9-2 and WX11-3 exhibit differences in DNA supercoiling. This latter possibility may account for pPM31 multimerization in strain WX11-3, since recombination is known to be affected by plasmid DNA supercoiling (Drlica, 1984). The multimerization of plasmids pDS1109 or RSF2124, would as expected, be

limited to a minimum as a consequence of the presence of the cer locus (Summers & Sherratt, 1984), and a transposon-encoded site-specific co-integrate resolution system (Hakkaart et al., 1982).

Differences in plasmid multimerization with respect to growth conditions, and host mutant background, may reflect differential expression of the SOS-induced RecF pathway of plasmid recombination (Kolodner et al., 1985). An observation that may support this postulate, is that multimerization of pWX9 in strain WX11-8 varies with incubation temperature, consistent with the temperature-dependent cell division defect of this strain.

Finally, DNA isolation and restriction analysis of plasmid dimers, suggests that they may be formed by head-to-tail covalent linkage of plasmid monomers. Indeed, site-specific interplasmidic recombination, possibly mediated by the RecF pathway (Kolodner et al., 1985), would be expected to lead to the formation of head-to-tail covalently linked plasmid dimers (Kennedy et al., 1983). Plasmid multimerization, therefore, may have been a major factor contributing to the segregational instability of plasmid-bearing cells during chemostat culture.

4.5 Chemostat culture of plasmid-bearing host mutant strains WX11-8 and WX9-2

4.5.1 Introduction

To confirm that for at least one of the mutant

strains the host defect represented a mutation that affected plasmid segregation, rather than a plasmid mutation that had given rise to a plasmid-free cell, plasmid-bearing strains of WX11-8, carrying either pDS1109, pWX9 or pWX11, and WX9-2 carrying pWX9, were assayed for plasmid maintenance during phosphate-limited chemostat culture (Fig.4.8).

4.5.2 Results

a) Plasmid stability during chemostat culture of strain WX11-8

At time₀, when continuous flow of medium was initiated, plasmid-free segregants were detected in all three chemostat cultures (Fig.4.8). Cells that had lost pDS1109 constituted an approximately 3% initial plasmid-free population, which within 40 generations had increased rapidly to about 96% of the culture.

In contrast, pWX11 and pWX9 exhibited a greater degree of persistence during chemostat culture (Fig. 4.8). Cells that had lost pWX11, significantly increased in number only after about 40 generations of continuous culture, constituting some 85% of the culture population at 100 generations. Cells that had lost pWX9, only increased in number following 60 generations of continuous culture, thereafter, plasmid-free segregants slowly increased comprising some 60% of the population at 140 generations.

Finally, during each of the three chemostat experiments, cells of strain WX11-8, when plated directly from the chemostat vessel onto rich medium,

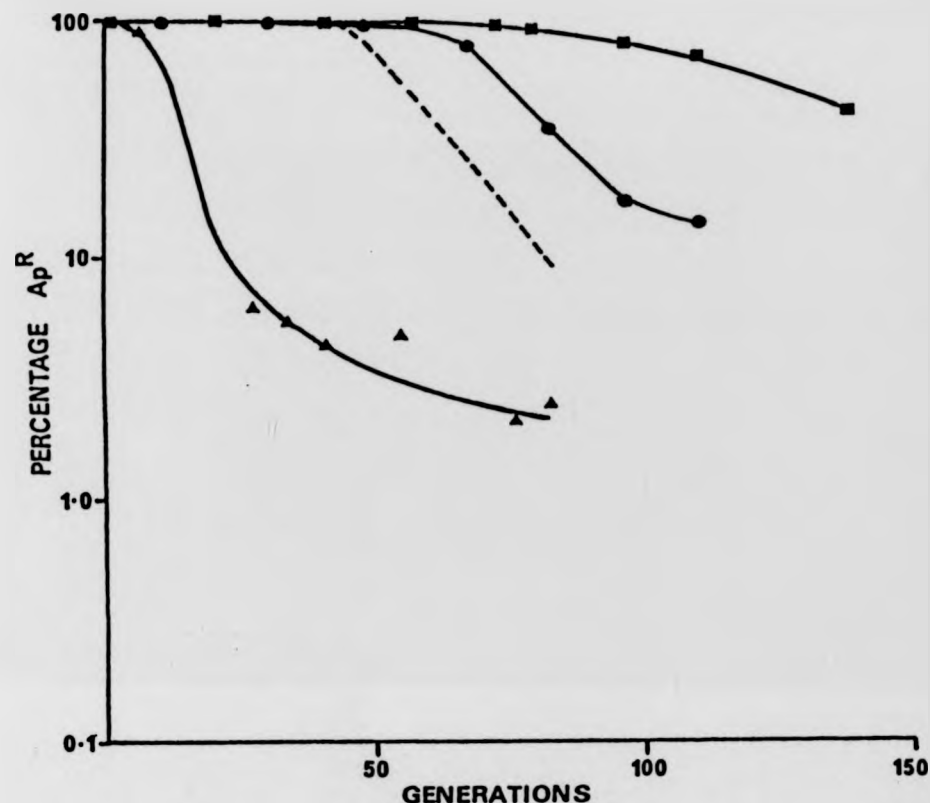


Figure 4.8

Persistence of plasmids pWX9, pWX11 and pDS1109 in strain WX11-8, during phosphate-limited chemostat culture. Results are presented as a semi-logarithmic plot of percentage ampicillin-resistant population with number of generations; ■—■, pWX9; ●—●, pWX11 and ▲—▲, pDS1109.

Data showing the persistence of plasmid pBR322 in *E. coli* strain W5445, during phosphate-limited chemostat culture;-----, is taken from Jones *et al.* (1980b). In all four experiments the dilution rate was 0.2hr^{-1} (a mean generation time of about 3.47hrs.).

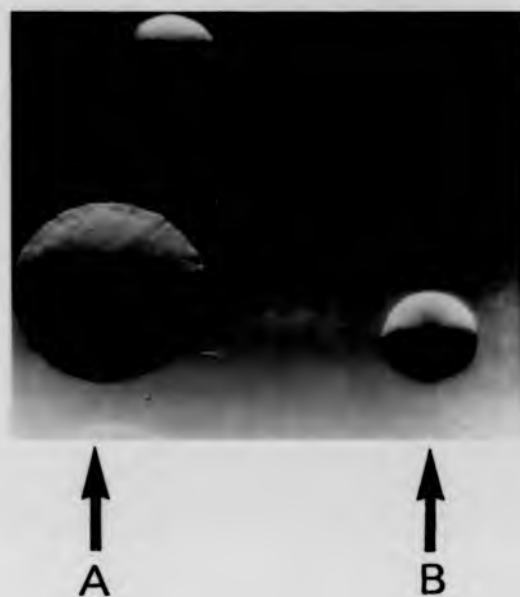


Figure 4.9

Colony morphologies of E.coli strain WX11-8 cells bearing plasmid pDS1109, when plated directly onto rich medium following about 35 generations of phosphate-limited chemostat culture.

A) characteristic large, circular 'pie crust' colony,

B) small, circular colony.

(Magnification 10-fold).

exhibited a mixture of two distinct colony morphologies (Fig.4.9). These were either small, circular, colonies possessing a convex elevation, or large, circular, raised colonies with a characteristic 'pie crust' outline. When tested for ampicillin resistance, no correlation appeared to exist between colony-type and antibiotic resistance. All colonies tested were resistant to streptomycin.

b) Chemostat culture of strain WX9-2 exhibits washout

Strain WX9-2 carrying pWX9 failed to establish a phosphate-limited chemostat culture when dilution rates of 0.2hr^{-1} , or 0.075hr^{-1} were used (Fig.4.10). Monitoring of cell volume changes via a Coulter counter and Channelizer, indicated that a block in the cell division process had occurred during transition from batch culture to nutrient limitation (Fig.4.11), resulting in a doubling of cell volume and consequent culture washout from the chemostat vessel. Towards the end of each washout period, only about 1.5% ($D=0.075\text{hr}^{-1}$), and 0.5% ($D=0.2\text{hr}^{-1}$), plasmid-free segregants were detected.

4.5.3 Concluding remarks

As alluded to previously, the four mutant strains appear to represent two distinct cellular morphologies. Strain WX11-8 produces filaments and minicells, while strain WX9-2 produces Y-shaped and filamentous cells. In addition, these two strains represent mutant

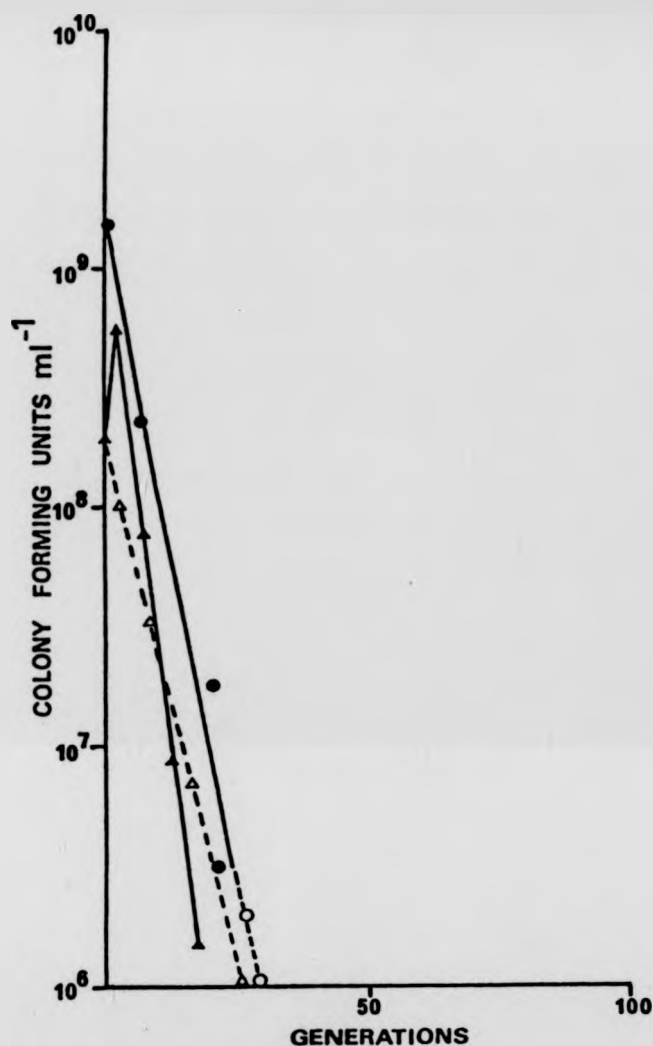


Figure 4.10

Actual and theoretical washout kinetics during phosphate-limited chemostat culture of *E.coli* strain WX9-2 bearing plasmid pWX9. Results are presented as a semi-logarithmic plot of colony forming units ml^{-1} with number of generations ; $\blacktriangle \longrightarrow \blacktriangle$, actual washout of cells at $D = 0.075 \text{ hr}^{-1}$; $\triangle \text{-----} \triangle$, theoretical washout for nondividing cells at $D = 0.075 \text{ hr}^{-1}$; $\bullet \longrightarrow \bullet$, actual washout of cells at $D = 0.2 \text{ hr}^{-1}$; $\circ \text{-----} \circ$, theoretical washout for nondividing cells at $D = 0.2 \text{ hr}^{-1}$.

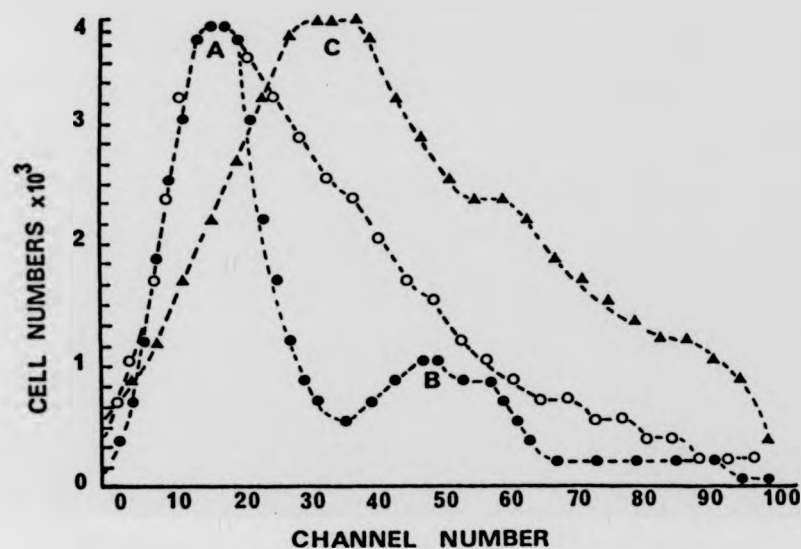


Figure 4.11

Changes in cell volume of *E.coli* strain WX9-2 bearing plasmid pWX9, during phosphate-limited chemostat culture at $D = 0.075\text{hr}^{-1}$. Cell volume distribution at commencement of media flow; ●-----●, following about 6 generations of medium flow; ○-----○, following about 8 generations of medium flow; ▲-----▲. Peak A = a cell volume of $0.64\mu\text{m}^3$, peak B = a cell volume of $1.28\mu\text{m}^3$, and peak C = a cell volume of $0.98\mu\text{m}^3$.

derivatives of W5445, that are phenotypically more stable than strains WX11-2 and WX11-3, which segregate nonmucoid colonies at an appreciable frequency. Choice of strains WX11-8 and WX9-2 for phosphate-limited chemostat culture, was therefore a consequence of experimental convenience.

Initial batch culture, prior to chemostat culture of plasmid-bearing cells of strain WX11-8, resulted in the generation of about a 3% subpopulation of plasmid-free cells. During the chemostat culture of pDS1109-bearing cells, these plasmid-free cells readily predominated. Since pDS1109 would not be expected to form multimers, leading to a high segregation rate for plasmid-free cells, then the rapid predominance of plasmid-free segregants may appear puzzling. However, inter- and intraplasmidic recombination of pDS1109 in strain WX11-8, may be expected to exist as a dynamic equilibrium, a situation that could lead to the segregation of plasmid-free cells at a low frequency. Rapid predominance of plasmid-free cells, may have occurred as a consequence of the postulated selective disadvantage conferred on pDS1109-bearing cells.

In contrast, both pWX11- and pWX9-bearing cells of strain WX11-8 co-existed for 40 and 60 generations respectively, with an approximately 3% subpopulation of plasmid-free cells. Enhanced multimerization of pWX9 and pWX11 in strain WX11-8 (Table 4.4 & Fig.4.5 C, lane d), may have been a major factor contributing to the generation of plasmid-segregants. However, the kinetics of plasmid-free cell predominance during chemostat

culture of pWX11-bearing cells, differed markedly to that occurring during the chemostat culture of pWX9-bearing cells, and suggests that plasmid-free cells in both chemostat experiments may have acquired some distinct selective advantage over their parent plasmid-bearing strain. In addition, the appearance of distinct colony types during the chemostat culture of plasmid-bearing cells of strain WX11-8, emphasizes the inherent phenotypic instability of the four mutant strains isolated.

Finally, it is evident from attempts to initiate chemostat culture of strain WX9-2, that mutants possessing similar cellular defects may not be maintained during chemostat culture. This may explain why such mutant cells appear to be at a selective disadvantage in establishing predominance over plasmid-bearing cells of strain W5445. Interestingly, the actual and theoretical washout kinetics for strain WX9-2 coincide at a dilution rate of 0.2hr^{-1} , strain WX9-2 was isolated at this dilution rate. A reduction in the dilution rate to 0.075hr^{-1} , delayed the onset of division inhibition by 2 to 3 generations, and this is reflected in the higher percentage of plasmid-free cells detected at this lower dilution rate. Since it has been determined that the sfiA-dependent pathway of division inhibition is not involved in either cell size determination, chromosome segregation, or size variation following nutritional shifts (Huisman et al., 1983), then division inhibition of strain WX9-2, following transition from batch to nutrient limitation, may

suggest the possibility of some defect(s) occurring in the sfi-independent pathway of division inhibition.

4.6 Discussion

4.6.1 Introduction

It is obvious that there are two fundamental differences between the plasmid-bearing host mutants and their plasmid-bearing parent, which appear to contribute to the segregational stability of ColE1-type plasmids. The first of these differences relates to some defect occurring in the cell division cycle. The second difference, a possible indirect result of the cell division defect, relates to an enhanced multimerization of plasmid molecules. Observations made during phenotypic characterization of the host mutants, provides some information towards a possible explanation of the nature of the cellular defect that alters the pattern of cell division, the means by which this defect may have been generated, and the consequences for plasmid recombination and segregational stability.

4.6.2 Hypothetical basis for the generation of mutants defective in cell division

As postulated in Chapter III, a high plasmid copy number, in addition to contributing to segregational plasmid stability, may also have resulted in the generation of host mutants. Strain WX11-8, as suggested by minicell formation, increased resistance to U.V.irradiation and novobiocin sensitivity, may possess

a defect in the GyrB subunit of DNA gyrase. Indeed, DNA gyrase, a component of the cellular DNA synthesizing machinery, is required by both plasmid and chromosome replication. The GyrB subunit of DNA gyrase has been shown to be essential for transcription coupled to dnaA-dependent initiation of chromosome replication (Filutowicz & Jonczyk, 1981), in addition to DNA polymerization (Filutowicz & Jonczyk, 1983). Initiation of ColEI-type replication has also been shown to be sensitive to both the superhelical density of the DNA template and the GyrB inhibitor novobiocin (Orr & Staudenbauer, 1981; Wolfson et al., 1982). Sequestration of DNA gyrase by a sufficient number of ColEI-type origins per chromosomal origin of replication, may have exerted intracellular pressure for changes in the affinity with which the enzyme binds to either plasmid or chromosomal DNA, altering the efficiency with which initiation of DNA replication or DNA polymerization occurs. Indeed, increases in plasmid copy number during chemostat culture of plasmid-bearing cells (Fig.3.13 & 3.23), may have been a consequence of the generation of host mutant cells possessing an altered ratio of cell volume to chromosomal DNA content. Plasmid pWX9, which increased substantially in copy number during chemostat culture (Fig.3.13), would appear to readily generate such host mutants. This suggests the possibility that the par sequence derived from plasmid pSC101, may have an affinity for the binding of DNA gyrase.

It is interesting to note that the gyrB gene, located on the E.coli genome close to oriC at

83 minutes, lies within a cluster of genes involved in DNA synthesis (Kuwabara & Uchida, 1981). The gene order is gyrB, recF, dnaN and dnaA. The dnaN gene codes for the beta-subunit of DNA polymerase III, an enzyme involved in DNA polymerization. The dnaE gene product, the alpha-subunit of DNA polymerase III, is required for both plasmid and chromosome replication and is known to interact with the dnaN gene product (Kuwabara & Uchida, 1981). Transcription of the dnaN gene, has been shown to occur predominantly from the dnaA gene promoter (Sakakibara *et al.*, 1981). Expression of the recF gene product, although produced in substantially different amounts to that of the dnaN gene product, is also expressed from a common promoter (Blancar *et al.*, 1984), which suggests that expression of the dnaA to recF gene cluster may be coordinately regulated.

The recF gene product has been postulated to have a function in controlling the normal cell division cycle (Thomas & Lloyd, 1983). During normal DNA replication, the recF gene product may function as a direct or indirect effector of recA activity, modulating expression of the recA/lexA controlled sfiA inhibitor of cell division. Interestingly, inhibition of the GyrB subunit by coumermycin A₁, leads to a recF-dependent induction of recA synthesis (Smith, 1983). It was concluded that some characteristic of the GyrB-coumermycin A₁ complex, rather than inhibition of DNA gyrase activity, was responsible for generating the recF-dependent recA-inducing signal. In contrast, the GyrA inhibitors, oxolinic acid and nalidixic acid, cause

a recF-independent induction of recA.

A mutational alteration of the GyrB subunit may, therefore, not only lead to defects in initiation of chromosome replication and DNA polymerization but could also result in a recF-dependent induction of the SOS response. Strains WX9-2, WX11-2 and WX11-3, however, do differ phenotypically with respect to strain WX11-8. The main differences occur in the cellular morphology they exhibit and the extent to which plasmid multimerization is enhanced. However, all strains show a similar pattern of sensitivity to inhibitors of DNA gyrase, which suggests these strains may also possess defects in DNA gyrase.

4.6.3 Plasmid multimerization

The major pathway for general recombination involving plasmids is that of RecF (Kolodner *et al.*, 1985), the expression of which is controlled as part of the recA/lexA regulon. Interestingly, sfrA mutations, that appear to be allelic with recA, suggest that the recF gene product may play a role(s) in modulating recA functions (Volkert & Hartke, 1984), leading to either rec-dependent recombination, presumably as a consequence of an alteration in recA recombinational activity, or induction of the SOS response, as a result of an alteration in recA protease activity. The link between recF-dependent induction of the SOS response and the GyrB subunit of DNA gyrase (Smith, 1983), may therefore account for the increase in plasmid multimerization, observed in concert with alterations in cell division.

However, only certain plasmid species exhibited an obvious increase in multimerization, a possible consequence of the nature of the DNA substrate for recombination. Indeed, it has been suggested that specific DNA sequences able to stimulate recombination, probably have a major effect on plasmid recombination. Studies in which DNA segments that stimulate recombination have been isolated, indicate that most of these elements act in cis, suggesting that plasmids generally undergo minimal recombination in the absence of such stimulatory sequences (James et al., 1983).

4.6.4 Conclusions

The basis for the observed differences in mucoidy, cell division, U.V. resistance and plasmid multimerization, between parent and plasmid-free mutant isolates of strain W5445, is concluded to be the consequence of a defect(s) in the functioning of DNA gyrase. An alteration in respect of DNA gyrase function(s), is postulated to lead to a partial induction of the SOS response. This is presumed to result in an induction of DNA repair mechanisms, the RecF pathway of plasmid recombination and the cell division inhibitor Sula. The sula gene product is presumed to interfere with the functioning of the essential cell division gene product ftsZ, leading to alterations in cell division and a mucoid colony phenotype. Plasmid multimerization, postulated to occur via the RecF pathway, is assumed to be mediated by specific DNA sequences able to stimulate recombination.

Finally, plasmid multimerization is concluded to have made a major contribution to the segregational instability of ColE1-type plasmids.

CHAPTER V

PLASMID MULTIMERIZATION AND THE SFI-DEPENDENT
PATHWAY OF DIVISION INHIBITION

5.1 Introduction

Host mutants defective in both plasmid segregation and cell division, and exhibiting multimer formation of certain plasmids, were isolated during chemostat culture studies designed to identify DNA sequences capable of conferring segregational stability on plasmid pBR322. Phenotypic characterization of these mutants indicated that similarities exist between them and lon strains.

The lon gene has been shown to encode the monomer component of the ATP-dependent protease, protease La (Schoemaker & Markovitz, 1981), whose proteolytic activity is increased on binding to single-stranded DNA (Charette et al., 1984). Mutations in the lon gene (capR or deg), effect a variety of cellular processes. lon strains have a decreased ability to degrade abnormal proteins, as well as certain wild-type proteins, they overproduce capsular polysaccharide, and have been shown to be defective in both plasmid maintenance and establishment, as well as lysogenization by bacteriophages lambda and P1 (Gottesman & Zipser, 1978; Markovitz, 1977; Falkinham, 1979; Walker et al., 1973; Takano, 1971). Transfer of lon strains from minimal to rich medium, inhibits cell division and promotes nonseptate filament formation (Berg et al., 1976). In addition, lon strains have an increased sensitivity to DNA damaging agents, such as U.V.-light or methyl methanesulphonate (MMS), that also induce filamentation (Berg et al., 1976; Johnson, 1977). Sensitivity to U.V.-light or MMS can be suppressed either chemically,

by post-irradiation incubation with the furan derivative DL-pantoyllactone (PL), (Kirby, et al., 1972; Nakayama et al.; 1982), or genetically with one of two additional mutations, sulA (sfiA) or sulB (sfiB), which map at distinct loci and prevent the lethal filamentation observed in lon strains (Gottesman et al., 1981b).

The sulA gene product, the expression of which is controlled by the LexA repressor, has been proposed to be a cell division inhibitor of the sfi-dependent pathway of division inhibition, induced following DNA damage (Huisman et al., 1983). Protease La, has been shown to affect the stability or activity of the sulA gene product (Schoemaker et al., 1984), which suggests that lon strains, in a response to DNA damaging agents, may accumulate the Sula polypeptide, resulting in filament formation. However, nutritional shifts do not lead to an induction of sulA gene expression (Huisman et al., 1983), which suggests that filamentation during a nutritional shift may not be the consequence of a derepression of the sfi-dependent pathway of division inhibition. Mutations in sulB occur at a lower frequency than those in sulA, and are allelic with mutations in ftsZ, an essential cell division gene the product of which is proposed to interact with the postulated Sula division inhibitor (Ward & Lutkenhaus, 1984). It was of some interest, therefore, to determine what effects a lon and lon sul background would have on plasmid integrity. The independently isolated E.coli K-12 lon strains PAM660 lon-22 (Johnson, 1977), and RGC103 capR9 (Gayda et al., 1976), were chosen for

investigation, together with derivatives of PAM660 carrying either a sulA (PAM163), or sulB (PAM161), extragenic suppressor mutation (Johnson, 1977). All strains used throughout this study (derivatives of either W5445, AB1157 or X156, a derivative of W945), were presumed to be derived from E.coli K-12 strain Y10 (Bachmann, 1972). Before plasmid analysis, the lon and lon sul derivatives, were phenotypically characterized.

5.2 Phenotypic characterization of lon and lon sul strains

5.2.1 Colony morphology

Colonies of the lon and lon sul strains PAM660, PAM161, PAM163 and RGCl03 all gave rise to mucoid colonies on A+B minimal agar at 30°C (Fig.4.1). The colonies of all strains on L-agar at 37°C, were easily distinguishable from the colonies of their respective parent strains. Both the lon-22 sul strains, PAM161 and PAM163, and the capR9 parent strain X156, gave rise to large, circular colonies possessing a characteristic 'pie crust' outline. The lon-22 strain PAM660, also gave rise to large, circular colonies, but these possessed a lobate margin rather than the characteristic 'pie crust' outline of the lon-22 sul derivatives. In contrast, both AB1157 and the capR9 strain RGCl03, gave rise to small, circular colonies having a convex elevation and a well defined margin.

Plating efficiencies of all strains on rich medium, following overnight growth in A+B minimal medium at

37°C, were markedly different (Table 5.1). The capR9 strain RGCl03 and its parent X156, both exhibited marked reductions in their plating efficiencies. Strain RGCl03, however, showed a greater reduction in plating efficiency than did X156. On the other hand, strains AB1157, PAM161 and PAM163 all gave similar efficiencies of plating, however, the lon sulA strain PAM163, did exhibit a slight decrease in its plating efficiency. In contrast, strain PAM660 failed to grow well in minimal medium. Large colonies which did arise on minimal agar were surrounded by micro-colonies, suggesting the occurrence of cross-feeding. This strain may possess a nutritional deficiency that is at present uncharacterized. Alternatively, the cross-feeding may have occurred as a consequence of the diffusion of a cytoplasmic membrane factor(s), associated with the ubiquinone-cytochrome-dependent respiratory chain, reported to promote septation in lon mutants following exposure to ionizing irradiation (Adler et al., 1981).

5.2.2 Cellular morphology

Phase-contrast microscope observations of bacterial cells, indicated that the cellular morphologies exhibited by all strains, could be conveniently categorized into three distinct classes. The cells of strains PAM163 and X156 at 37°C, are somewhat similar in morphology to those of AB1157 (i.e., rod-shaped), however, both PAM163 and X156 cells are capable of forming moderately lengthy nonseptate filaments. Indeed, at 42°C cells of strain X156 do form nonseptate

<u>Strain</u> <u>designation</u>	<u>Relevant</u> <u>genotype</u>	<u>Plating efficiencies</u>	
		<u>minimal medium</u>	<u>rich medium</u>
AB1157	<u>lon</u> ⁺	1	1.02
PAM660	<u>lon</u> -22	-	ND
PAM161	<u>lon</u> -22, <u>sul</u> B-25	1	0.96
PAM163	<u>lon</u> -22, <u>sul</u> A-27	1	0.81
X156	<u>azi</u> , <u>cap</u> R ⁺	1	0.36
RGC103	<u>azi</u> , <u>cap</u> R9	1	0.06

Table 5.1

The efficiencies of plating onto rich medium of E.coli strains AB1157, PAM161, PAM163, X156 and RGC103, following overnight growth in A+B minimal medium at 37°C. Plating efficiencies were calculated as the average of two separate platings. The plating efficiency of E.coli strain PAM660 could not be determined because of insufficient growth in minimal medium; ND = not determined.

filaments, presumably as a consequence of the azi mutation (Yura & Wada, 1968). On the other hand, cells of strain PAM163 do not filament at 42°C, but exhibit irregular pleomorphic shapes.

Both the lon-22 and capR9 cells of strains PAM660 and RGCl03 respectively, form long nonseptate filaments at 37°C. However, the filaments formed by strain PAM660 are not as extensive as those formed by RGCl03.

In contrast, cells of strain PAM161 do not form filaments at either 37°C or 42°C, however, Y-shaped cells can be observed as a proportion of the population at 37°C, and their numbers increase with growth at 42°C.

5.2.3 U.V.-light resistance and antibiotic sensitivity

Sensitivity to MMS, the radiomimetic agent nitrofurantoin and U.V.-light irradiation are characteristic phenotypes of lon strains (Johnson, 1977; Kirby *et al.*, 1972; Berg *et al.*, 1976). Strains PAM660 and RGCl03 were sensitive to MMS at 30°C, 37°C and 42°C, while the parent strains, AB1157 and X156 were resistant at both 30°C and 37°C, but showed a partial sensitivity at 42°C (Table 5.2 A). Suppressors of lon, designated sul, are selected as a consequence of their resistance to MMS (Johnson, 1977). Strain PAM163 was found to have the same pattern of resistance to MMS as strain AB1157, while strain PAM161, although resistant at 30°C and 37°C, was nevertheless sensitive to MMS at 42°C (Table 5.2 A).

Sensitivity to nitrofurantoin was exhibited by strains PAM660 and RGCl03 at 30°C and 37°C (Table

A)

Strain designation:	<u>Methyl methanesulphonate</u>			<u>Nitrofurantoin</u>			<u>Sodium azide</u>			<u>Relevant genotype</u>
	30°C	37°C	42°C	30°C	37°C	42°C	30°C	37°C	42°C	
XL56	+	+	(+)	+	+	+	+	+	+	<u>azi</u> , <u>capR</u> ⁺
RGCL03	-	-	-	-	-	-	+	+	+	<u>azi</u> , <u>capR</u> ⁹
AB1157	+	+	(+)	+	+	+	-	-	-	<u>lon</u> ⁺
PAM660	-	-	-	-	-	+	-	-	-	<u>lon</u> -22
PAM161	+	+	-	-	+	-	-	-	-	<u>lon</u> -22, <u>sulB</u> -25
PAM163	+	+	(+)	-	+	-	-	-	-	<u>lon</u> -22, <u>sulA</u> -27

B)

<u>Strain designation:</u>	<u>GyrA subunit</u>						<u>GyrB subunit</u>						<u>Relevant genotype</u>
	<u>Nalidixic acid</u>			<u>Oxolinic acid</u>			<u>Novobiocin</u>			<u>Coumermycin A₁</u>			
	30°C	37°C	42°C	30°C	37°C	42°C	30°C	37°C	42°C	30°C	37°C	42°C	
XL56	-	-	-	-	-	-	-	-	-	-	-	-	<u>azi</u> , <u>capR</u> ⁺
RGCL03	-	-	-	-	-	-	-	-	-	-	-	-	<u>azi</u> , <u>capR</u> ⁹
AB1157	-	-	-	-	-	+	-	-	-	-	-	-	<u>lon</u> ⁺
PAM660	-	-	-	-	-	+	-	-	-	-	-	-	<u>lon</u> -22
PAM161	-	-	-	-	-	(+)	-	-	-	-	-	-	<u>lon</u> -22, <u>sulB</u> -25
PAM163	-	-	-	-	-	(+)	-	-	-	-	-	-	<u>lon</u> -22, <u>sulA</u> -27

Table 5.2

Sensitivity of the growth of *E. coli* strains AB1157, PAM660, PAM161, PAM163, XL56 and RGCL03 on L-agar at 30°C, 37°C and 42°C, to the antimicrobial compounds, A) methyl methanesulphonate (250µl/litre), nitrofurantoin (4µg/ml), and sodium azide (100µg/ml), B) nalidixic acid (50µg/ml), oxolinic acid (0.5µg/ml), novobiocin (500µg/ml), and coumermycin A₁ (20µg/ml). +, = growth; -, = no growth; (+), = sparse growth.

5.2 A), however, in contrast to RGCl03, PAM660 was resistant to nitrofurantoin at 42°C. Both parent strains, AB1157 and X156, were resistant to nitrofurantoin at 30°C, 37°C and 42°C, while strains PAM161 and PAM163 were resistant at 37°C only, being sensitive both at 30°C and 42°C (Table 5.2 A).

During exponential growth, both lon and capR9 strains exhibited a similar sensitivity to U.V.-light irradiation, as may have been expected of class A lon mutants (Donch & Greenberg, 1968), while the respective parent strains, AB1157 and X156, were substantially more resistant to U.V. irradiation (Fig. 5.1 A, a). On the other hand, exponentially growing cells of strains PAM161 and PAM163, exhibited a sensitivity to U.V.-light irradiation lower than that of their parent strain PAM660, but slightly greater than that of AB1157 (Fig. 5.1 A, b). Both strains, however, did not exhibit similar U.V.-light sensitivities, PAM161 was more resistant than PAM163 to U.V.-light irradiation. Furthermore, the addition of PL to the post-irradiation plating medium, increased substantially the survival of both strains PAM660 and RGCl03 to U.V.-light irradiation (Fig. 5.1 B), a further characteristic feature of lon strains (Donch & Greenberg, 1968).

Strain W5445 was previously found to be resistant to the gyrB inhibitor novobiocin, and to exhibit a temperature-dependent resistance to the gyrA inhibitors nalidixic acid and oxolinic acid (Table 4.2 B, see Chapter IV). Since all of the above strains are assumed to be related to strain W5445, the resistance of these

Figure 5.1

Sensitivity to U.V.-light irradiation of exponential phase cells of *E.coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103. Results are presented as semi-logarithmic plots of the percentage colony forming units surviving increasing periods of U.V.-light irradiation measured in Joules m^{-2} .

A) Percentage survival U.V.-light irradiation following overnight incubation at 37°C on L-agar:

a) strains AB1157; \square — \square , PAM660; \blacksquare — \blacksquare ,
X156; \circ — \circ , and RGC103; \bullet — \bullet .

b) strains AB1157; \square — \square , PAM660; \blacksquare — \blacksquare ,
PAM161; \bullet — \bullet , and PAM163; \blacktriangle — \blacktriangle .

B) Percentage survival U.V.-light irradiation following overnight incubation at 37°C on L-agar and L-agar containing DL-pantoyllactone, strains PAM660; \blacksquare — \blacksquare , and RGC103; \bullet — \bullet , incubated on L-agar, and PAM660; \square — \square , RGC103; \circ — \circ , incubated on L-agar containing DL-pantoyllactone.

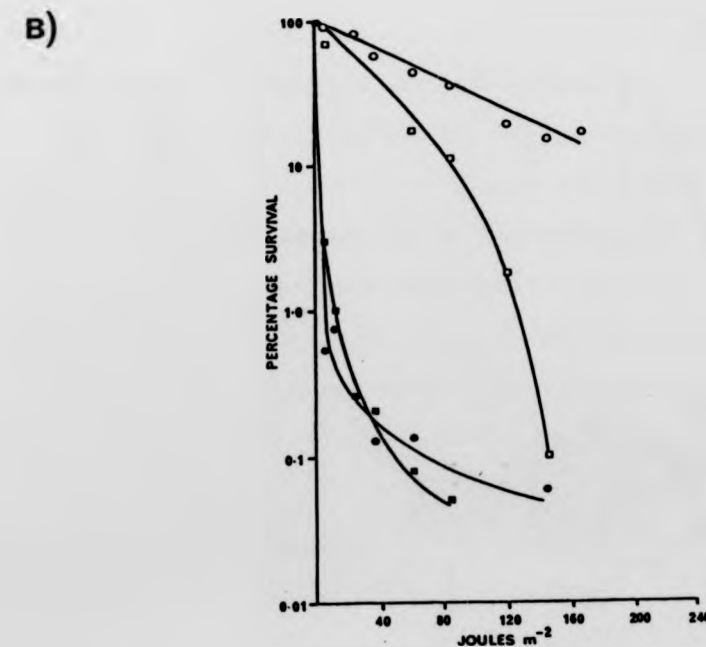
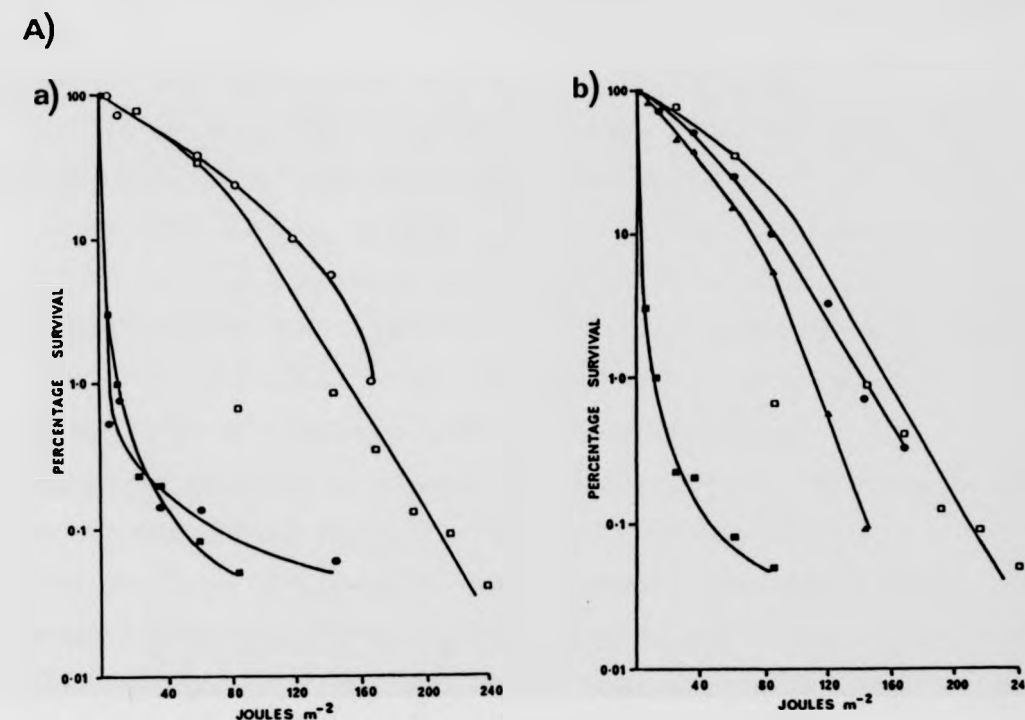


Figure 5.1

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation. The percentage of cells surviving irradiation is plotted against the dose of 0.5 V-light irradiation in Joules cm^{-2} .

At 30°C, strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 were all sensitive to 0.5 V-light irradiation. At 37°C, strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 were all sensitive to 0.5 V-light irradiation. At 42°C, strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 were all sensitive to 0.5 V-light irradiation.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

Survival of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 following 0.5 V-light irradiation at 30°C, 37°C and 42°C.

Legend: AB1157 (○), PAM660 (●), PAM161 (△), PAM163 (□), X156 (◇), RGC103 (×).

strains was determined with respect to the *gyrA* inhibitors nalidixic acid and oxolinic acid, and the *gyrB* inhibitors novobiocin and coumermycin A₁.

Strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103, all proved to be sensitive to novobiocin, coumermycin A₁ and nalidixic acid at all temperatures tested (Table 5.2 B). On the other hand, all strains except X156 and RGC103, exhibited a temperature-dependent resistance to oxolinic acid at 42°C. Strains AB1157 and PAM660 were sensitive to oxolinic acid at both 30°C and 37°C, while strains PAM161 and PAM163 were sensitive at only 30°C. Interestingly, light microscope observations of PAM161 and PAM163 indicated that the cells of these strains form extensive filaments in the presence of oxolinic acid at 37°C, but not at 42°C. This may explain why both strains gave only sparse growth on oxolinic acid at 37°C, possibly the consequence of lethal filament formation.

Finally, strains X156 and RGC103 specify resistance to sodium azide at 30°C, 37°C and 42°C (Table 5.2 A). Sodium azide resistance is a phenotype associated with the formation of filamentous cells at 42°C (Yura & Wada, 1968). In contrast, all other strains proved to be sensitive to sodium azide at all temperatures tested (Table 5.2 A).

5.2.4 Concluding Remarks

Close examination of the phenotypes of both *lon* and *lon sul* strains, reveals a similarity between strains WX9-2, WX11-2 and WX11-3, and strains carrying defects

in the sfi-dependent pathway of division inhibition (i.e., PAM161 lon sulB and PAM163 lon sulA). Hence, the characteristic 'pie crust' colonies on rich medium, mucoid colonies on minimal medium (Fig.4.1), and sensitivity to MMS at 42°C of strains PAM161 and PAM163 (Table 5.2 A), together with Y-shaped cell formation in strain PAM161, are all phenotypic similarities shared with strains WX9-2, WX11-2 and WX11-3 (Fig.4.1 & Fig.4.2; Table 4.2 A).

It is evident that both lon and lon sul strains exhibit a greater sensitivity to U.V. irradiation than their wild-type parent strain AB1157 (Fig.5.1 A). This contrasts markedly with the increased U.V. resistance of strains WX9-2, WX11-2 and WX11-3, in comparison to their parent strain W5445 (Fig.4.3 A). This would suggest that it is the presence of a lon mutation that confers sensitivity to U.V. irradiation. Indeed, the presence of PL in the post U.V. irradiation plating medium restored U.V. resistance to the lon strains RGC103 and PAM660 (Fig.5.1 B), presumably as a consequence of the suppression of the lethal effects of induced filament formation (Kirby *et al.*, 1972).

Resistance of strain W5445, to antibiotics which inhibit subunit functions of DNA gyrase, was a characteristic difference between this strain and its plasmid-free host mutant derivatives (Table 4.2 B). The wild-type parents, their lon derivatives and the lon sul strains, do not exhibit a similar pattern of resistance or sensitivity, particularly with respect to novobiocin (Table 5.2 B). This would suggest that the resistance

of strain W5445 to novobiocin, reflects a genuine defect in its DNA gyrase, and is not a consequence of cell envelope exclusion of the antibiotic.

Curiously, oxolinic acid induced the formation of filamentous cells in the lon sul strains PAM161 and PAM163 at 37°C. It may be that oxolinic acid interferes with some aspect of the initiation of chromosome replication or segregation, leading to inhibition of cell division via the sfi-independent pathway. Indeed, the effects of oxolinic acid on strain PAM161 and PAM163, contrasts with the suppression of temperature induced filament formation observed in cells of strain WX11-8 at 42°C. Strain WX11-8 is presumed to carry a gyrB^{ts} mutation, binding of oxolinic acid to the gyrA subunit of DNA gyrase may have suppressed a functional defect(s) in the DNA gyrase enzyme. Indeed, it has been demonstrated that an alteration in one subunit of DNA gyrase, may allosterically affect the other subunit (Yamagishi *et al.*, 1981; Bogdanova *et al.*, 1982).

5.3 Effect of lon and lon sul mutations on plasmid configuration

5.3.1 Introduction

Plasmid-free mutants of strain W5445 which arose during chemostat culture of pWX9- and pWX11-bearing cells, exhibited certain phenotypic characteristics in common with strains known to carry mutations in genes encoding the sfi-dependent pathway of division inhibition. In addition, these spontaneously arising

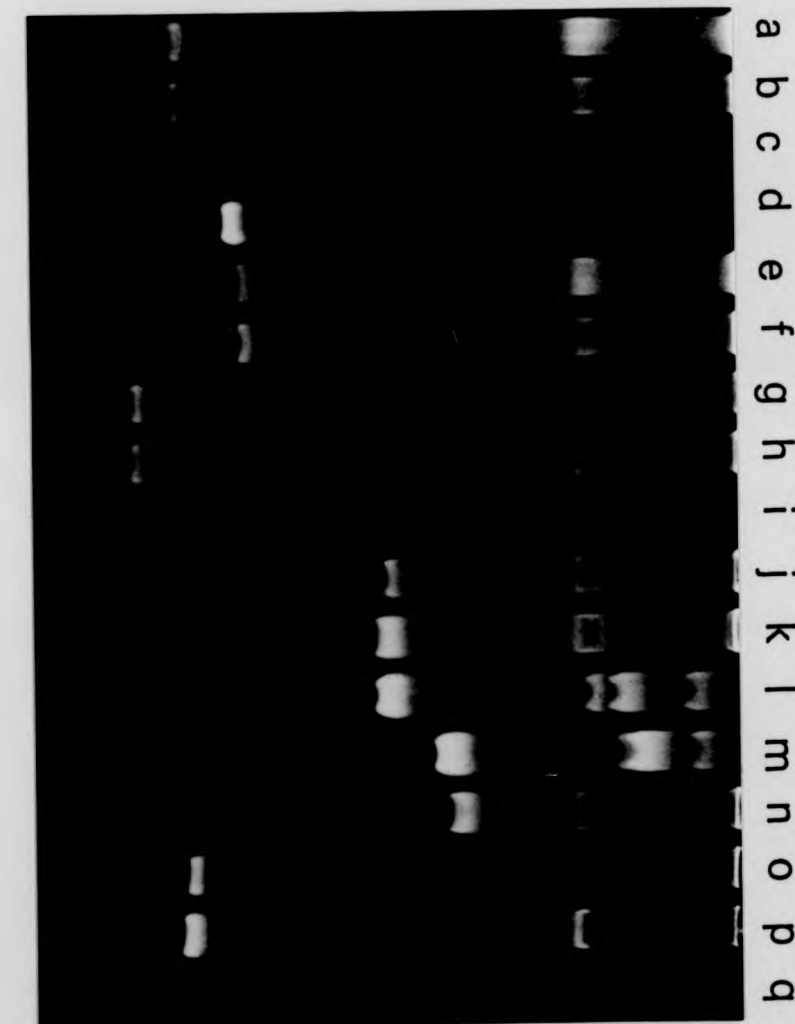
Figure 5.2

Representative agarose gel electrophoresis of multimeric plasmid DNA profiles, obtained by rapid, small-scale plasmid preparations of *E.coli* K-12 strains, cultured at 37°C under ampicillin selective pressure.

A) Plasmid pWX9 isolated from, lanes a and b) strains PAM660 and AB1157 respectively grown in rich medium, o and p) strains PAM660 and AB1157 respectively grown in minimal medium, e and f) plasmid pBR322 isolated from strains PAM660 and AB1157 respectively grown in rich medium, g and h) plasmid pPM31 isolated from strains PAM660 and AB1157 respectively grown in minimal medium, j and k) plasmid pOU93 isolated from strains PAM660 and AB1157 respectively grown in minimal medium, c, d, i, l and q) purified plasmid DNA preparations of pWX9, pBR322, pPM31, pOU93 and pWX9 respectively isolated from strain W5445 following CsCl-EtBr density gradient centrifugation, m) purified plasmid pWX9-8 DNA isolated from WX11-8 following CsCl-EtBr density gradient centrifugation, n) plasmid pWX9-8 isolated from strain AB1157 grown in minimal medium.

B) Plasmid pWX9 isolated from, lanes a and b) strains RGC103 and X156 respectively grown in minimal medium, d and e) plasmid pBR322 isolated from strains RGC103 and X156 respectively grown in minimal medium, c and f) purified plasmid DNA preparations of pWX9 and pBR322 respectively isolated from strain W5445 following CsCl-EtBr density gradient centrifugation.

A)



B)

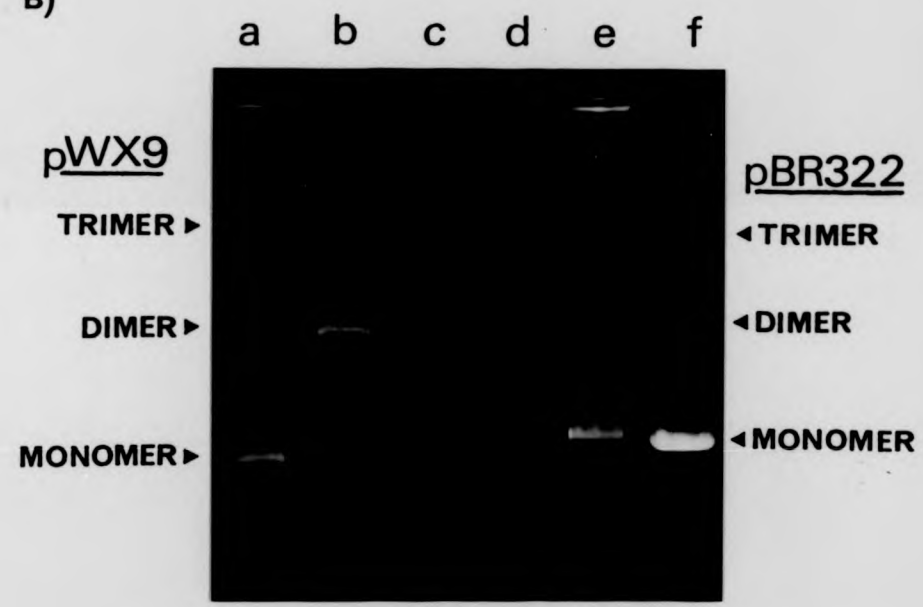
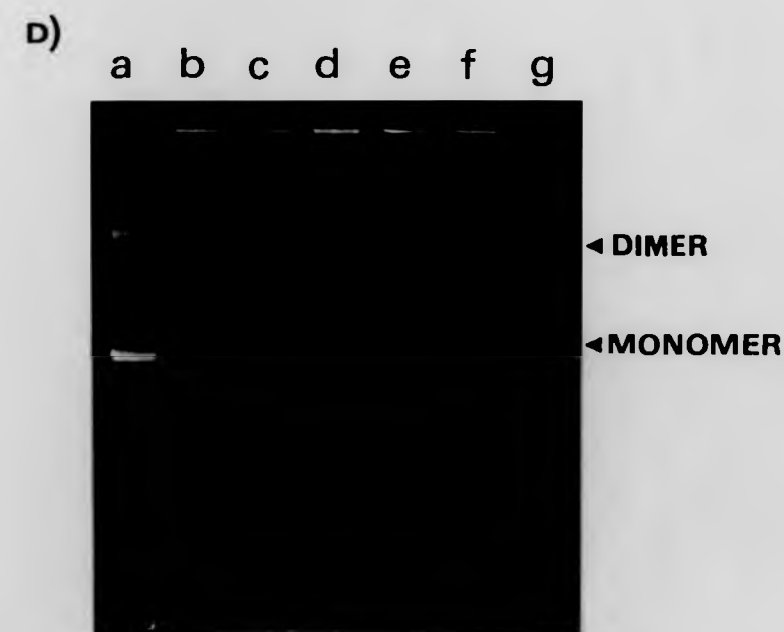
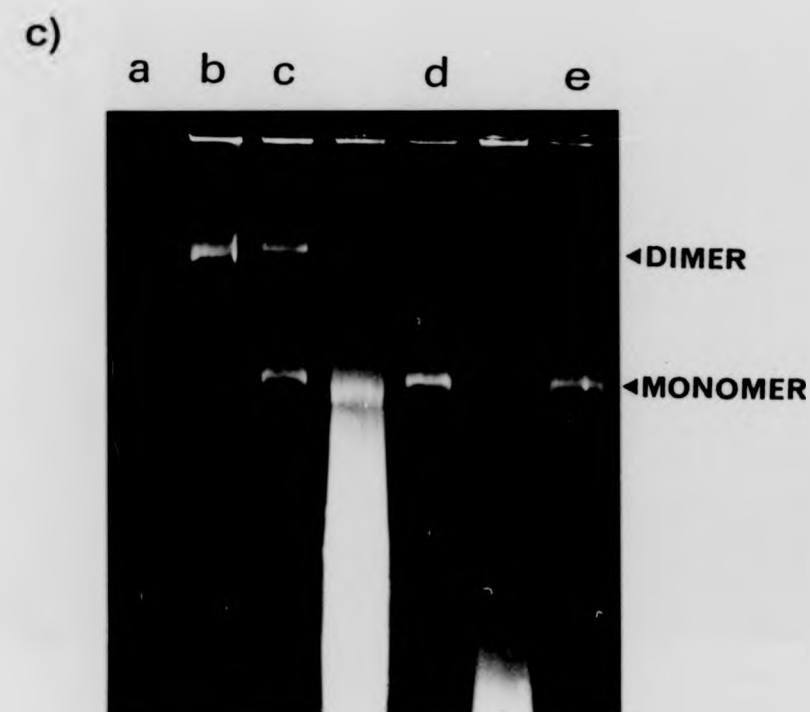


Figure 5.2 Con't.,

C) Lane a) purified plasmid DNA preparation of pPM31, isolated from strain W5445 following CsCl-EtBr density gradient centrifugation, b to e) plasmid pPM31 isolated from respectively, strains PAM163 and PAM161 grown in minimal medium, and strains AB1157 and WX9-2 (see Chapter IV), grown in rich medium.

D) Plasmid pWX11 isolated from, lanes b to d and g) strains PAM161, RGCl03, X156 and W5445 respectively grown in minimal medium, e and f) strains WX11-3 and WX11-2 respectively grown in rich medium (See Chapter IV), a) purified plasmid pWX11 isolated from strain W5445 following CsCl-EtBr density gradient centrifugation.



<u>Strain designation</u>	<u>Plasmid designation</u>										<u>Relevant genotype</u>
	<u>minimal medium</u>					<u>rich medium</u>					
	<u>pBR322</u>	<u>pWX9</u>	<u>pWX11</u>	<u>pPM31</u>	<u>pOU93</u>	<u>pBR322</u>	<u>pWX9</u>	<u>pWX11</u>	<u>pPM31</u>	<u>pOU93</u>	
AB1157	M	M	M	M	M	M	M	M	M	M	<u>lon</u> ⁺
PAM660	M	M	M	M	M	M	M	M	M	M	<u>lon</u> -22
PAM161	M	M	D	M	D	M	M	D	M	D	<u>lon</u> -22, <u>sulB</u> -25
PAM163	M	T	D	D	M	M	D	D	D	M	<u>lon</u> -22, <u>sulA</u> -27
X156	M	D	D	M	M	M	D	D	M	M	<u>azi</u> , <u>capR</u> ⁺
RGCL03	M	M	D	M	M	M	M	D	M	M	<u>azi</u> , <u>capR9</u>

Table 5.3

Configuration of the lowest, clearly observable plasmid multimer for plasmids pBR322, pWX9, pWX11, pPM31 and pOU93, as determined by migrational analysis in agarose gels following rapid, small-scale plasmid isolation from transformants of *E. coli* strains AB1157, PAM660, PAM161, PAM163, X156 and RGCl03. Transformants were incubated at 37°C under appropriate antibiotic selective conditions, in either minimal or rich medium; M = monomer; D = dimer; T = trimer.

plasmid-free mutant segregants also possessed an enhanced ability to form multimers of certain recombinant plasmid derivatives, a factor which may have contributed to destabilization of the respective recombinant plasmid. It was of interest, therefore, to ascertain whether strains possessing mutations in genes encoding the sfi-dependent pathway of division inhibition, would likewise enhance multimer formation of these same plasmids, and whether the pattern of multimerization was in anyway similar to that in the plasmid-free mutant segregants of strain W5445.

5.3.2 Results

a) Plasmid multimerization

Strains AB1157, PAM660, PAM161, PAM163, X156 and RGC103 were transformed with plasmid pBR322, pWX9, pWX11, pPM31 and pOU93 DNA and their configurations compared by agarose gel analysis following rapid, small-scale plasmid isolation.

When grown in either minimal or rich medium, all plasmids isolated from strains AB1157 and PAM660, were found to exist mainly as monomers (Fig.5.2 A & Table 5.3), as did plasmid pBR322 on isolation from all other transformant strains (Fig.5.2 A & B; Table 5.3). In contrast, PAM161, PAM163, X156 and RGC103, when grown in either minimal or rich medium gave rise to dimers of pWX11 (Fig.5.2 D, lanes b to d; Table 5.3 & Fig.4.5 C, lanes b & c, Chapter IV). Dimer formation of plasmids pOU93 in PAM161, pPM31 in PAM163 (Fig.5.2 C, lane b), and pWX9 in X156 (Fig.5.2 B, lane b), was also found to

occur when these strains were grown in either minimal or rich medium (Table 5.3). However, when PAM163 carrying pWX9 was grown in minimal medium, the lowest multimer observable was a trimer (Fig.4.7 A, lane f, Chapter IV), while growth in rich medium gave rise to dimers (Table 5.3). Finally, it was noted that plasmids isolated from strain RGC103, appeared to exhibit a slightly increased apparent molecular weight, as determined by gel electrophoresis, in comparison to plasmids isolated from the other strains (Fig.4.5 C, lane c; Fig.4.5 E, lane a, Chapter IV & Fig.5.2 B, lane d).

b) Characterization of plasmid dimers

Plasmid pWX9 DNA was isolated and purified by CsCl-EtBr density gradient centrifugation, following chloramphenicol amplification, from strain PAM161 grown in minimal medium, and strain PAM163 grown in rich medium and minimal medium containing PL (Fig.5.3). Addition of PL to the minimal growth medium of strain PAM163 bearing plasmid pWX9, appears to increase the amount of pWX9 dimer in comparison to trimer isolated. EcoRI restriction analysis, suggests that pWX9 isolated from strain PAM163, exists as a covalently linked head-to-tail dimer, when compared with EcoRI generated restriction fragments produced by monomeric pWX9 DNA isolated from strain PAM161 (Fig.5.3). However, it is evident that the pattern of EcoRI restriction of pWX9 DNA isolated from strains PAM161 and PAM163 (Fig.5.3), differs in comparison to the pattern of EcoRI restricted pWX9 DNA isolated from strains W5445 and WX11-8



Figure 5.3

Comparative EcoRI restriction endonuclease analysis of purified multimeric plasmid pWX9 DNA preparations, harvested from CsCl-EtBr density gradients. Lanes a and b) undigested and digested pWX9-163 DNA, isolated from strain PAM163 grown in minimal medium containing DL-pantoyllactone, which appears to enhance the amount of pWX9 dimer, c and d) undigested and digested pWX9-163 DNA isolated from strain PAM163 grown in rich medium, e and f) undigested and digested pWX9 DNA isolated from strain PAM161.

(Fig.4.6, Chapter IV). This difference appears to be the consequence of an increase in the size of pWX9 DNA isolated from strains PAM161 and PAM163. This increase is estimated to be the result of an approximately 1kb insert.

5.3.3 Concluding remarks

Plasmid DNA isolation and restriction analysis would appear to confirm that plasmid multimers, isolated from strains carrying defects in the sfi-dependent pathway of division inhibition, possess a covalently linked head-to-tail configuration (Fig.5.3). It is apparent that following transformation into strains PAM161 and PAM163, insertion of additional DNA into plasmid pWX9 occurred, an event that may have promoted plasmid multimerization. However, it is also evident that the lon-22 host background of strain PAM660 does not result in an increase in plasmid multimerization (Fig.5.2 A; Table 5.3). And that strains PAM161, PAM163 and X156 which possess some phenotypic similarities with strains WX9-2, WX11-2 and WX11-3, all gave rise to plasmid multimers, particularly of plasmids pWX9 and pWX11 (Fig.4.5 C, Chapter IV & Fig.5.2 C; Table 5.3), suggesting that the pathway by which interplasmidic recombination occurs in these strains may be similar to that in the four mutant derivatives of strain W5445. It would appear, therefore, that a mutation in either sulA, sulB or azi, which may lead to an imbalance in the normal cell division cycle, may result in an induction of the SOS response and therefore the RecF pathway of

plasmid recombination (Kolodner, et al., 1985).

The azi capR9 strain RGCl03, contrasts with its azi parent strain X156 in the multimerization of pWX9 (Table 5.3). The presence of the capR9 mutation appears to suppress multimerization of pWX9 (Fig.5.2 B, lanes a & b). The capR9 mutation may be expected to lead to an increase in the concentration or activity of the division inhibitor Sula (Schoemaker et al., 1984). Curiously, the lon sula mutant strain PAM163, exhibited plasmid multimer formation to a greater extent than that of the other strains (Table 5.3). This may suggest that plasmid multimer formation may be indirectly dependent on the concentration or activity of a functional sula gene product. The Sula polypeptide may therefore be important in co-ordinating the cell division cycle. Alternatively, the slight increase in the apparent molecular weight of plasmids isolated from RGCl03, as determined by gel electrophoresis, may suggest that either plasmid DNA supercoiling is altered in this host background, or that plasmids insertionally acquire additional DNA. Both these possibilities may alter the potential for plasmid multimerization.

5.4 Discussion

5.4.1 Introduction

A great deal of interest has been shown in the use, for the commercial production of cloned gene products, of host strains that carry a lon background. This interest, stems from a desire to reduce losses in

product yield as a result of proteolysis. However, the use of lon or lon sul derivatives may pose certain problems.

5.4.2 Plasmid multimerization

It is evident that strains carrying defects in the cell division cycle, or the sfi-dependent pathway of division inhibition, are likely to exhibit enhanced plasmid multimerization, as a possible consequence of the induction of the RecF pathway. Multimerization of certain plasmid species, may be expected to lead to segregational plasmid instability. Hence, care should be exercised when choosing a suitable host background for the commercial production of a cloned gene product, particularly with respect to mutations associated with the sfi-dependent pathway of division inhibition.

5.4.3 Structural plasmid instability

Restriction endonuclease analysis of pWX9 DNA, isolated from strains PAM161 and PAM163, indicated the presence of an approximately 1kb of additional DNA. Furthermore, plasmids isolated from strain RGCl03, migrated during agarose gel electrophoresis with slightly increased apparent molecular weights, also a possible consequence of insertional DNA. Acquisition of DNA may occur as a consequence of either general recombination, or transposition. This would suggest, therefore, that host backgrounds derived from either strains AB1157 or X156, may pose problems with respect to both the structural and segregational instability of

recombinant plasmids.

5.4.4 Prospects for continuous culture

Extragenic suppression of lon by sul mutations may be expected to lead to strains which behave in a manner similar to strain WX9-2. Although strain WX9-2 was able to grow in batch culture, transition to chemostat culture resulted in division inhibition and washout from the chemostat vessel (Chapter IV). This suggests that variations in culture conditions may have detrimental effects on strains phenotypically similar to WX9-2. This poses the question as to whether it may be possible to continuously culture lon sul strains.

5.4.5 Conclusions

It is postulated that plasmid-bearing cells, derived from either AB1157 or X156, which carry defects in either the cell division cycle or the sfi-dependent pathway of division inhibition, may exhibit both structural and segregational plasmid instability, as a consequence of insertional DNA rearrangement and plasmid multimerization. Finally, it is proposed that continuous culture of such strains may not be possible, as nutrient limitation may lead to division inhibition and culture washout.

CHAPTER VI

FUTURE RECOMMENDATIONS AND PROSPECTS

6.1 Introduction

In retrospect, plasmids would appear not to be simple 'silent' passengers within bacterial cells, but capable of interacting in either a 'symbiotic' or 'parasitic' fashion with their host. Plasmid-host interactions which affect specific growth-rate, plasmid copy number, or DNA rearrangements, brought about by pathways of recombination or the transposition of mobile elements, have a direct bearing on the persistence and structural stability of plasmids within bacterial populations.

Continuous culture is an invaluable technique for the study of plasmid-host interactions, particularly with respect to plasmid stability. In addition to the easy maintenance of pH and dissolved oxygen concentration, the chemostat enables control of two important culture parameters, a limiting nutrient and specific growth-rate. Chemostats can be left unattended for several days, they enable an accurate estimation of the number of generations through which cells have transpired, and they rarely experience problems of contamination. In addition, they may also provide a means for the selection and isolation of host variants, defective in chromosome-encoded functions associated directly or indirectly with plasmid maintenance. In essence, the chemostat circumvents the problem of plasmid-bearing cells alternating between culture conditions that could lead to fluctuations in specific growth-rate, reproductive fitness, ColE1-type plasmid copy number, or plasmid multimerization. Therefore,

chemostat culture should enable both quantitative and qualitative information to be obtained, concerning plasmid-host interactions.

6.2 Recommendations

With hindsight, future chemostat experiments designed to elucidate factors affecting plasmid stability should include techniques able to determine, a) plasmid copy number, b) changes in restriction endonuclease digest pattern, c) the ability of a plasmid to confer a reproductive advantage or disadvantage, and d) cell volume distribution and numbers.

Changes in plasmid copy number and restriction digest pattern, can be determined using standard techniques of plasmid DNA isolation and gel electrophoresis. The reproductive fitness of a plasmid-bearing cell can be ascertained following chemostat culture competition experiments, between essentially isogenic plasmid-free and plasmid-bearing strains. A Coulter counter and Channelizer will monitor cell volume distribution and numbers. Finally, viable count determinations and scoring of antibiotic markers, should be carried out using plating medium of a similar composition to that employed for continuous culture.

6.3 Future prospects

As with most experimental endeavours, more questions are posed than answered, and this study is of no exception. Three avenues for further experimental investigation are suggested, although with more time and

thought others will most certainly become apparent.

6.3.1 Genetic characterization of host mutants defective in plasmid segregation

Phenotypic characterization of host mutant plasmid-free segregants of strain W5445, suggests that a defect(s) in the functioning of DNA gyrase may account for the alterations observed in cellular morphology and plasmid multimerization. DNA gyrase is composed of two subunits, GyrA and GyrB, encoded by distinct loci at 48 minutes and 82 minutes respectively on the E.coli genome (Drlica, 1984). Results indicate that the parent strain W5445, may also encode an alteration in one of the subunits of DNA gyrase, most likely the GyrB subunit. The question arises as to the nature of this mutational difference and the subsequent mutational change(s) giving rise to derivatives of W5445, exhibiting alterations in cell division and plasmid recombination. It would therefore be necessary in the first instance, using either techniques of generalized transduction or conjugation, to confirm that a defect(s) in one or both subunits of DNA gyrase indeed constitutes the mutational basis for the observed phenotypic changes. Secondly, genetic and biochemical characterization of any mutational defect(s), could then be carried out using standard cloning techniques. Having confirmed and identified any mutational differences, questions arising may relate to how these differences affect functions associated with chromosome and ColE1-type replication, as well as cell division and plasmid multimerization.

The intracellular titration of DNA gyrase, by a high number of ColEl-type plasmid replicons, is postulated to have generated the defect(s) in DNA gyrase. It would be of some interest, therefore, to determine the relationship between ColEl-type plasmid copy number, and the limits to which a host cell can tolerate titration of components of its DNA synthesizing machinery. This may effectively be answered by the use of continuous culture techniques, in conjunction with techniques able to determine plasmid copy number and cell volume distribution. Of prime importance may be the choice of plasmid and host cell background. This raises the question as to whether host backgrounds, other than that of strain W5445, are able to generate similar host mutant plasmid-free segregants, or is W5445 predisposed by virtue of the already existent phenotypic difference in its DNA gyrase. In addition, it was suggested that the par region of plasmid pSC101 may specify a region that binds DNA gyrase. This possibility could be resolved, by the use of techniques already developed for the identification of such binding sites.

Finally, using techniques of continuous culture, it may be possible to select for and isolate host mutants defective in functions mediating plasmid partitioning, or cell division coupling to plasmid replication. Consideration may have to be given to the use of a suitable mutagen, and care should be taken to select a low- or intermediate-copy number plasmid encoding an appropriate reproductive disadvantage.

6.3.2 Structural and functional analysis of plasmid sequences

Sequences that specify plasmid-encoded stability functions, or that confer a reproductive advantage or disadvantage, or are able to mediate plasmid recombination, have a marked affect on the stability and maintenance of plasmids within bacterial populations.

This study attempted to determine what contribution par functions, derived from plasmids pSC101 and R1, could make to ColE1-type plasmid segregational stability. Several complicating factors, however, hindered any conclusive interpretations. In retrospect, the study of plasmid-encoded stability functions during chemostat culture would be more effective using a low- or intermediate-copy number replicon, encoding an appropriate reproductive disadvantage. Genetic manipulation of sequences specifying plasmid-encoded stability functions, in concert with continuous culture techniques, should aid their analysis.

Plasmid pDS1109 was postulated to confer a reproductive disadvantage, assumed to be encoded by ColE1 sequences. That a naturally occurring plasmid may encode a reproductive disadvantage, is a phenomenon less well understood than conferred reproductive advantages. It would be of some interest, therefore, to identify and characterize this ColE1 function.

Mobile genetic elements able to mediate plasmid DNA rearrangements, have a major effect on the structural stability of plasmids. In addition to mobile elements that are plasmid-encoded, elements present within host

backgrounds may also lead to problems of structural or segregational plasmid instability. Indeed, it was postulated that plasmid pWX15 may have acquired an insertional copy of the transposon Tn10, present within strain WX100. Likewise, plasmid pWX9 was postulated to have acquired a lkb insert from the host background of strains PAM161 and PAM163. Sequence insertions may not only result in an alteration of the integrity of the plasmid, but also plasmid copy number or functions associated with plasmid maintenance. Characterization of plasmid DNA rearrangements, originating from host backgrounds, and their effects on the structure and function of plasmid molecules, would be of some interest when considerations have to be made with respect to the choice or design of a suitable host strain.

Plasmid-encoded sequences, other than mobile genetic elements, that mediate plasmid multimerization were also postulated to have a significant effect on the segregational stability of plasmids. During this study, certain derivatives of plasmid pBR322 were shown to exhibit enhanced plasmid multimerization. It was postulated that sequences encoded within these derivatives, mediate plasmid multimerization via a host-encoded recombinational pathway(s). Identification of both the sequence(s) and the pathway(s) of interplasmidic recombination, could most readily be accomplished using standard cloning techniques, in conjunction with host strains possessing genetic lesions in appropriate pathways of recombination. Subsequent studies may then be carried out to determine how such

sequences function as substrates for recombination.

6.3.3 Plasmid segregation and the cell division cycle

Plasmid-bearing cells encoding defects in components of their cell division machinery, were observed to exhibit an increased potential for plasmid multimerization. Plasmid multimerization is undoubtedly a major factor, contributing to the segregational instability of plasmids. However, the contribution that a cell division defect may make to plasmid instability is not at present clear, particularly with respect to strains harbouring defects in the sfi-dependent pathway of division inhibition. For instance, how would such defects affect maintenance functions encoded by the F plasmid or pSC101? Indeed, would it be possible to use continuous culture techniques to investigate plasmid stability in lon sul strains, or would such strains exhibit washout, as did the host mutant WX9-2? And what might be the effect of introducing recA mutations into such strains, to reduce induction of the SOS system? These are interesting questions, answers to which may provide a better understanding of how plasmids relate or interact with their host cell.

Finally, it is apparent that a good deal of experimental understanding is lacking with regard to how certain chemical agents, particularly DL-pantoyllactone, methyl methanesulphonate, nitrofurantoin or sodium azide, interact biochemically with the cell division process. Indeed, the function encoded by the azi locus remains uncharacterized (Yura & Wada, 1968).

6.4 Implications for industrial biotechnology

In addition to satisfying academic curiosity, it was also envisaged that this study may be applicable to both present and future problems associated with the new emerging biotechnology industry. This new industry, based on the technology of genetic manipulation, may consider as options for fermentation either batch, continuous or air-flow cultures, as well as immobilized cell systems. However, if the stability of the biological system can be ensured, then more than likely any industrial fermentation process may achieve some degree of success. Although precise recommendations cannot be outlined, since the design, fermentation and purification of individual cloned gene products may each present their own intrinsic problems, guidelines may nevertheless be put forward that may limit problems of instability associated with recombinant strains.

Considerations with respect to both plasmid and host background are required. Ideally, plasmid copy number should be maintained at a low level during early stages of scale-up to production, to avoid any intracellular burden. For ColE1-type plasmids, this may involve the use of temperature-sensitive copy number replicons. A low plasmid copy number, however, will necessitate the use of some strategy to ensure segregational stability. A small, simple, plasmid-encoded stabilizing function, such as the par region of plasmid pSC101, may be a more suitable strategy than alternatives which make use of auxotrophic complementation. Plasmid-encoded sequences that may

mediate DNA rearrangements, plasmid multimerization or confer a reproductive disadvantage, should be avoided. However, the expression of a cloned gene product may itself confer a reproductive disadvantage, and therefore some form of regulated expression would be a desirable feature.

A suitable host background, carrying genetic lesions in functions encoded by lon and components of the RecF pathway, other than recF itself, may likewise promote the stability of a recombinant plasmid, in addition to its product, as would avoidance of host strains encoding mobile genetic elements. The use of recA strains, to minimize plasmid multimerization, may however, not be a realistic alternative, since both recA and lexA mutants are known to prematurely initiate cell division, resulting in the productin of cells smaller than wild-type, usually containing no DNA (Inouye, 1971; Howe & Mount, 1978).

Finally, although the biotechnology industry may use an assortment of fermentation technologies, the techniques of continuous culture would appear to represent a useful approach to the assessment of any biological system designed for the production of a cloned gene product.

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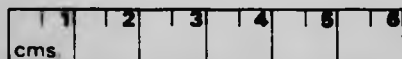
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